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Vegetalization of the Sea-Urchin Egg by Dinitrophenol and Animalization by Trypsin and Ficin

by SVEN HÖRSTADIUS[1]

From the Zoological Institute, Uppsala

HERBST (1892) made the remarkable discovery that the differentiation of the sea-urchin egg was shifted in a vegetal direction when lithium ions were present in the sea-water. The vegetalization implies a failure of the apical tuft to appear, a displacement of the skeleton-forming cells in the animal direction, a reduction of the ectodermal region, and a corresponding enlargement of the endoderm, which often leads to exogastrulation. Strong lithium action may lead to complete endodermization of the egg. Many other substances have later been found to cause a change of differentiation, either in a vegetal or animal direction, e.g. a partial or complete animalization by treatment of unfertilized eggs with SCN- or I-ions (also SO_4, Br, and tartrate, Lindahl, 1936). The animal and vegetal principles are considered as two opposite, antagonistic gradients (Runnström, 1928 *a, b*) representing different types of metabolism (Lindahl, 1936).

It is of particular interest to study the effect on determination of substances which are known to interfere with metabolism in a special way. This paper will deal with the action of dinitrophenol (DNP) which acts as an inhibitor of oxidative phosphorylation, and the proteolytic enzymes trypsin and ficin.

MATERIALS AND METHODS

The experiments were made on eggs of *Paracentrotus lividus*. The DNP used was α-DNP and β-DNP from George T. Gurr, London, and γ-DNP from E. Merck, Darmstadt. Cleavage is immediately brought to a standstill when eggs are put into a strong solution of DNP (Clowes & Krahl, 1936, and others). Concentrated solutions are toxic. The following concentrations were found suitable: a $\frac{1}{10}$-saturated solution of α-DNP in sea-water, a $\frac{1}{4}$-saturated solution of β-DNP, and a $\frac{1}{2}$- or $\frac{1}{4}$-saturated solution of γ-DNP. The treatment as a rule took place in the 16-cell stage, before the operation when animal and vegetal halves were separated, or also immediately after the operation. In some experiments the eggs were instead put in the DNP immediately after fertilization. The results were, as we shall see, very varied, but, as far as I can judge, it did not matter

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whether the eggs were treated after fertilization, shortly before the operation, or immediately after it. The time of treatment was as a rule 2–4 hours, sometimes 6 hours, but this duration as well as longer treatments often proved disastrous, the eggs sooner or later disintegrating. A shorter treatment also often caused some mortality.

The reason for using not only whole eggs but also animal and vegetal halves is that a slight shifting of the determination in the animal or vegetal direction is difficult to register with certainty in whole eggs, whereas the animal halves are particularly sensitive in this respect. In the absence of influence from the vegetal half, the animal half as a rule shows a more animal differentiation than its prospective significance. The material of the animal half normally gives rise to about two-thirds of the ectoderm, forming an apical tuft and later pavement epithelium, ciliated band, and stomodaeum. An isolated animal half does not gastrulate, nor does it form skeletal spicules. Furthermore, in isolated animal halves the apical tuft is as a rule more or less enlarged and neither ciliated band nor stomodaeum are formed because of the lack of sufficient vegetal influence. In some halves the apical tuft is more or less typical. Such halves may develop a ciliated band and even a stomodaeum. Animal halves from the same batch of eggs as a rule differentiate in a more or less uniform way, giving either the former, animal type, or the latter, vegetal type, or intermediate types.

Trypsin was used in concentrations of 0·1 or 0·05 per cent. and on halves applied after isolation, or on whole eggs after fertilization, for about 20 hours. Ficin was used in a concentration of 0·04 per cent.

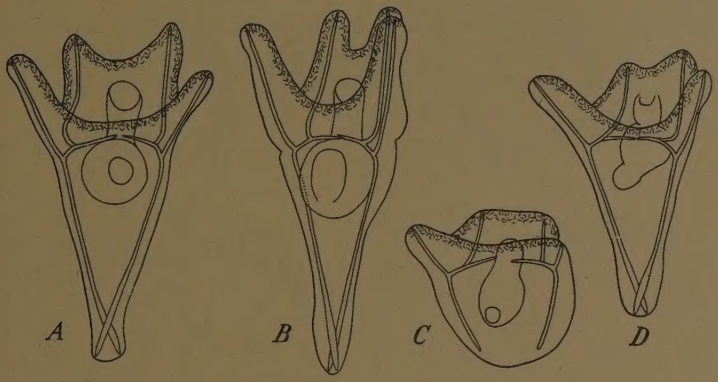
VEGETALIZATION BY DINITROPHENOL

The first experiments with DNP were very puzzling. In order not to use too many watchglasses in the preliminary series I put 5 whole eggs, 5 animal, and 5 vegetal halves in the same dish. In the first series I obtained in one dish one pluteus and one larva of the prism stage type among the animal halves, whereas the other animal halves remained in the usual way as blastulae. I thought I had made some mistakes in the operations. The pluteus might have emerged from a lateral half and the prism larva could have developed from a fragment with a few vegetal cells added to the animal cells. This interpretation, however, seemed very unlikely as I have isolated thousands of animal and vegetal halves without such operational mistakes. In the next series I took particular care to check the origin of the halves. Nevertheless several plutei, prism larvae, or gastrulae with spicules appeared among the animal halves. It was therefore evident that the DNP had a strong vegetalizing effect on some individuals, whereas other animal halves, vegetal halves, and whole eggs seemed to remain unaffected. These preliminary observations called for more detailed investigations.

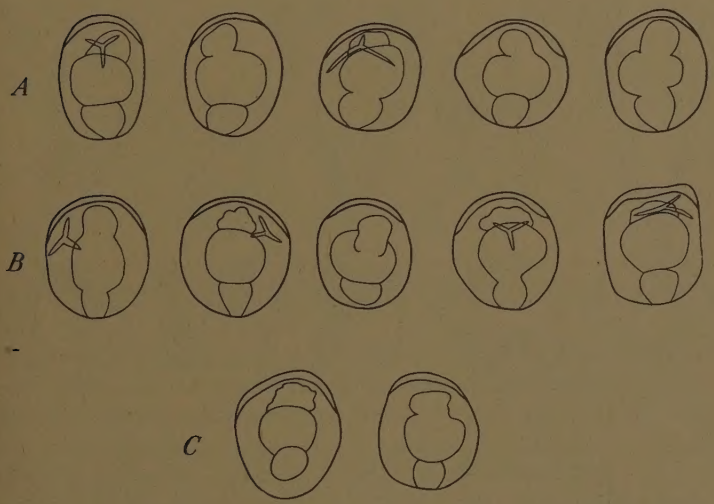
γ-DNP

I begin by describing the experiment 1950: 40 which gave the clearest result.

The concentrations used were $\frac{1}{4}$ or $\frac{1}{2}$ of a saturated solution and are in what follows referred to merely as $\frac{1}{4}$ or $\frac{1}{2}$.



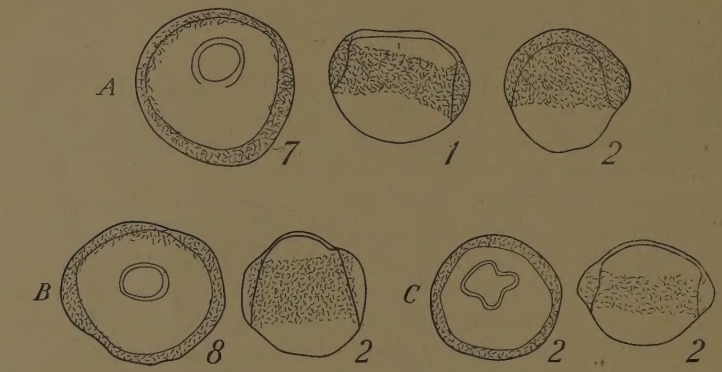
TEXT-FIG. 1. Whole eggs treated with a $\frac{1}{2}$-saturated solution of γ-DNP in sea-water. A, B, for 2 hours, C, D, for 4 hours. (Exp. 50:40.)



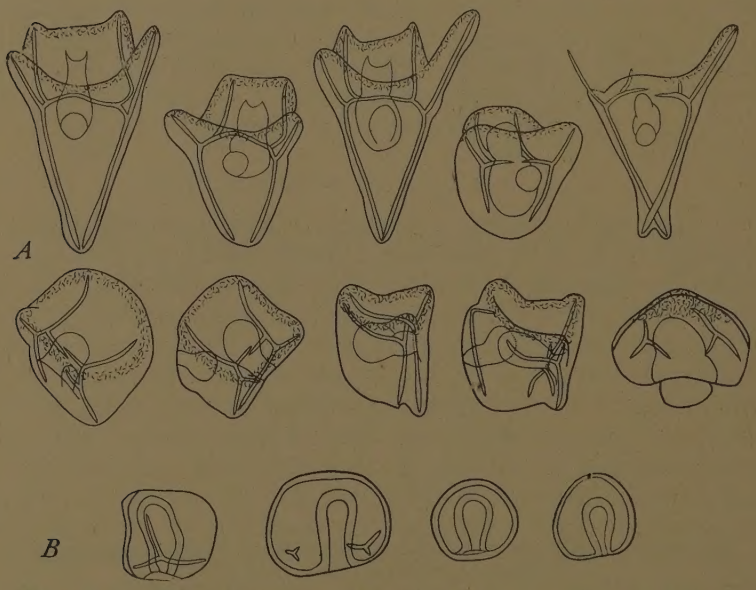
TEXT-FIG. 2. γ-DNP, $\frac{1}{4}$-saturated solution. Vegetal halves. A, controls. B, treated for $2\frac{1}{4}$ hours. C, treated for 4 hours. (Exp. 50:40.)

Whole eggs which had been lying 2 hours in $\frac{1}{2}$ developed into normal plutei (Text-fig. 1 A, B) and a treatment of 4 hours (C, D) also resulted in plutei, although not quite as typical as the former. We cannot, however, say that their differentiation is shifted in the animal or vegetal direction.

Vegetal halves treated for $2\frac{1}{4}$ hours (Text-fig. 2B) and 4 hours (Text-fig. 2C) with $\frac{1}{4}$ showed no difference when compared with the control halves (Text-fig. 2A).



TEXT-FIG. 3. γ-DNP, $\frac{1}{4}$-saturated solution. Animal halves. The figures give the number of halves of the respective types. A, controls. B, treated for $2\frac{1}{4}$ hours. C, treated for 4 hours. (Exp. 50 : 40.)

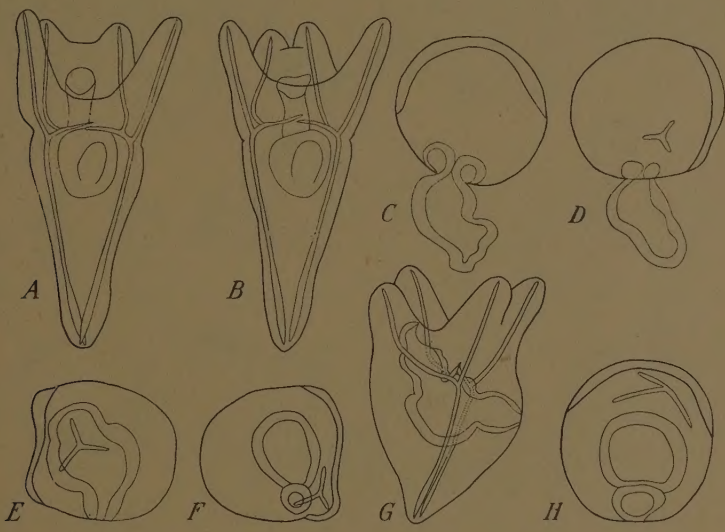


TEXT-FIG. 4. γ-DNP, $\frac{1}{2}$-saturated solution. Animal halves. A, treated for $2\frac{1}{2}$ hours. B, treated for 4 hours. (Exp. 50 : 40.)

But it is important to note that all the vegetal halves which had been lying $2\frac{1}{2}$ or 4 hours in $\frac{1}{2}$ were reduced or dead.

Examining the animal halves we find that $\frac{1}{4}$ for $2\frac{1}{4}$ hours (Text-fig. 3B) and

$\frac{1}{4}$ for 4 hours (Text-fig. 3C) caused no difference when compared with the controls (Text-fig. 3A). The picture is quite different when we compare the same controls (Text-fig. 3A) with the animal halves $\frac{1}{2}$ for $2\frac{1}{2}$ hours (Text-fig. 4A) and $\frac{1}{2}$ for 4 hours (Text-fig. 4B). In the former series all 10 halves have been transformed into plutei or larvae more or less resembling plutei with the exception of one which is still more vegetalized, forming a partial exogastrula. The 4 surviving after 4 hours in DNP (Text-fig. 4B) were gastrulae of a rather vegetal type, resembling vegetal halves.



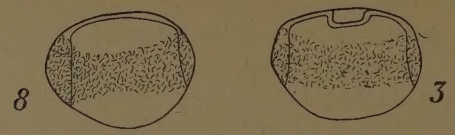
TEXT-FIG. 5. γ-DNP. Whole eggs. A–D, $\frac{1}{4}$-saturated solution for $1\frac{1}{2}$ hours. E, F, $\frac{1}{2}$-saturated solution for $1\frac{1}{2}$ hours. G, H, $\frac{1}{2}$-saturated solution for 3 hours. (Exp. 50:38.)

This experiment has shown that $\frac{1}{4}$ did not have any effect, but $\frac{1}{2}$ caused a strong vegetalization of animal halves and also death in vegetal halves. At the same time no influence upon determination in whole eggs could be detected.

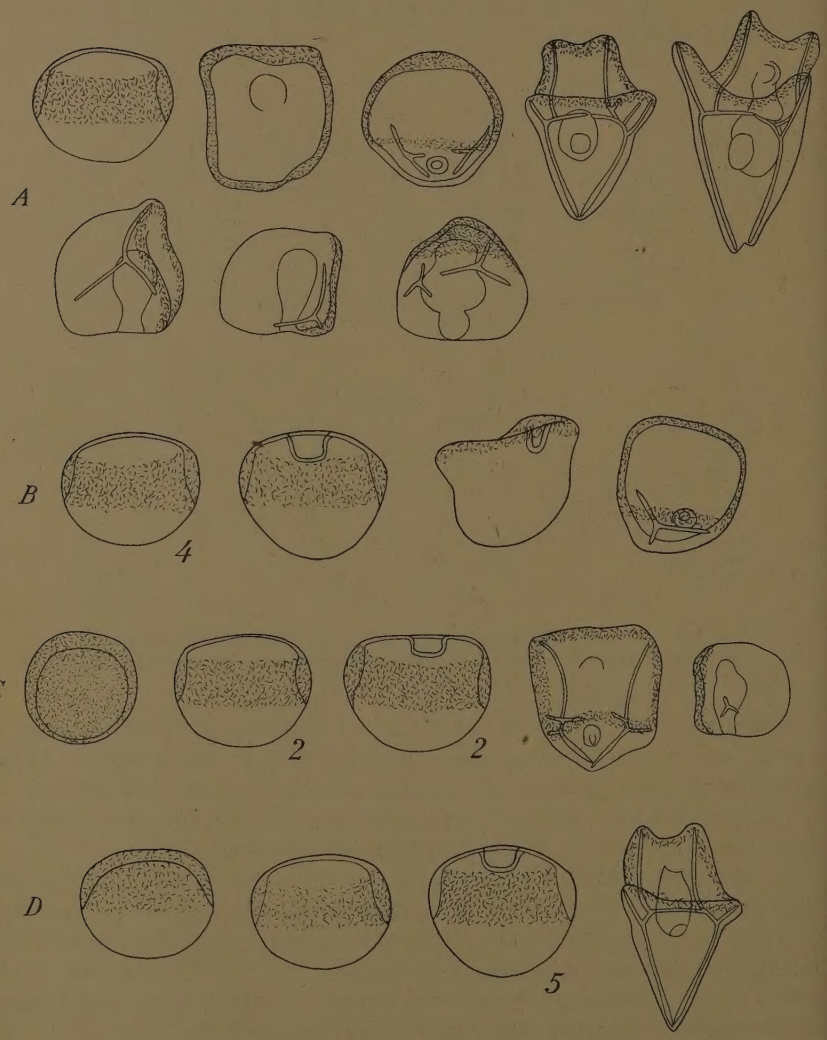
The conditions are, however, much more complicated than they appear in the above experiment, as will be seen from the following.

In experiment 1950:38 some also of the whole eggs became more or less vegetalized. Some differentiated as normal plutei after $1\frac{1}{2}$ hours in $\frac{1}{4}$ solution (Text-fig. 5 A, B), but others exogastrulated (C, D) and it seems fairly certain that endodermization has taken place in these cases. In $\frac{1}{2}$ for 3 hours plutei could still develop (Text-fig. 5G), but several larvae in $\frac{1}{2}$ for $1\frac{1}{2}$ hours and 3 hours closely resembled isolated vegetal halves (Text-fig. 5 E, F, H).

The animal controls produced 8 blastulae with a ciliated band and 3 with both band and stomodaeum (Text-fig. 6).

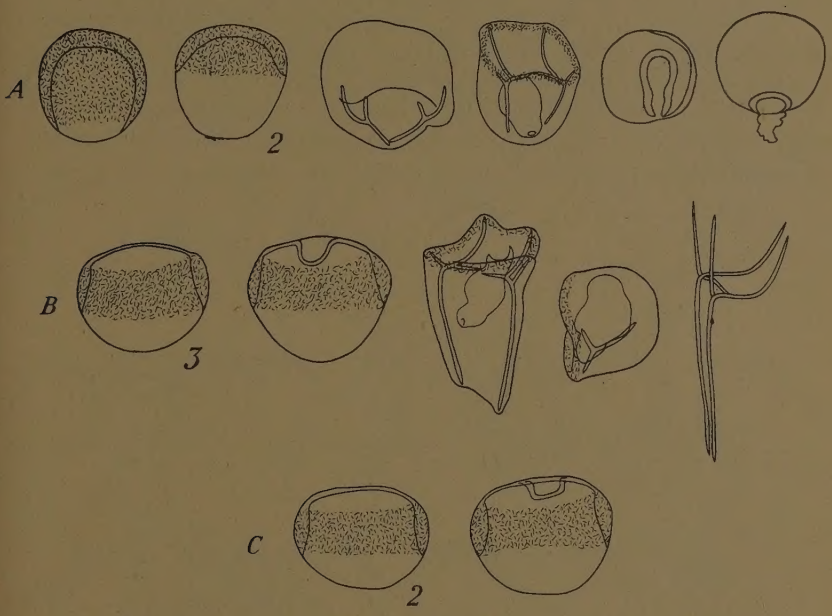


TEXT-FIG. 6. Animal halves, controls. (Exp. 50:38.)



TEXT-FIG. 7. γ-DNP, ¼-saturated solution. Animal halves. A, treated for 1½ hours before operation; B, treated for 1½ hours after operation; C, D, treated for 3 hours before operation. (Exp. 50:38.)

In the following series 10 animal halves were treated with DNP before or after operation. The surviving halves are shown in Text-figs. 7 and 8. After $1\frac{1}{2}$ hours in $\frac{1}{4}$ before operation (Text-fig. 7A), only 2 did not gastrulate. One formed a small archenteron and 2 triradiate spicules, 2 produced dwarf plutei, and 3 corresponded to prism stages or a vegetal half. With $\frac{1}{4}$ for $1\frac{1}{2}$ hours after operation (Text-fig. 7B) 6 developed as blastulae and 1 had a small archenteron and a



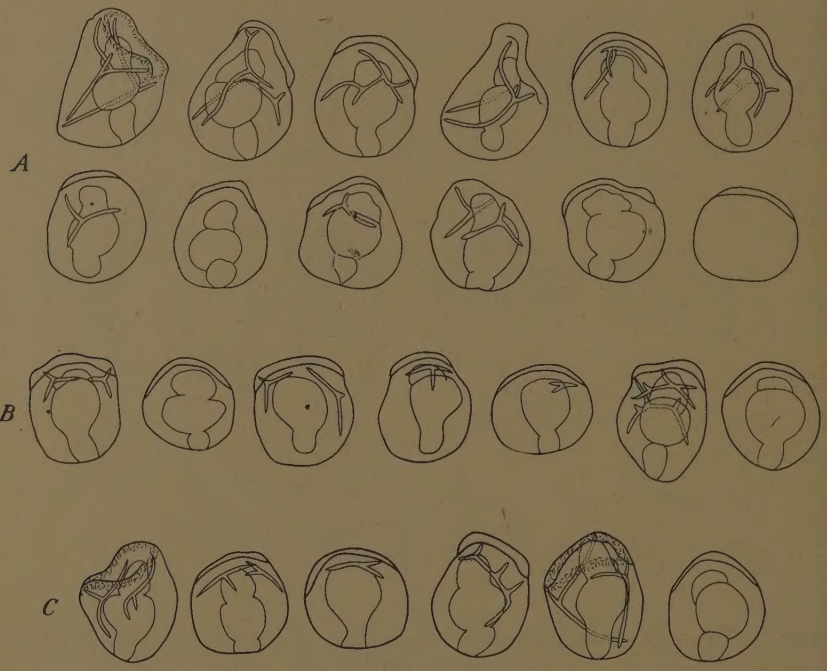
TEXT-FIG. 8. γ-DNP, $\frac{1}{2}$-saturated solution. Animal halves. A, B, treated for $1\frac{1}{2}$ hours. C, treated for 3 hours. (Exp. 50:38.)

spicule. It is strange that after 3 hours in $\frac{1}{4}$ before operation, the effect was less pronounced than after $1\frac{1}{2}$ hours: 5 blastulae, 1 larva with small archenteron and spicules, and 1 gastrula-prism stage (Text-fig. 7C). The same treatment after operation resulted in 7 blastulae and only 1 vegetalized half, but this one formed a real pluteus (Text-fig. 7D).

In the stronger solution ($\frac{1}{2}$) $1\frac{1}{2}$ hours before operation the experiment resulted in 3 blastulae without skeletons, 1 blastula with a skeleton, 1 prism larva, 1 gastrula, and 1 partial exogastrula without a skeleton (Text-fig. 8A). The corresponding series treated after operation (Text-fig. 8B) showed 4 blastulae, 1 pluteus with short arms, 1 fine pluteus (skeleton found stuck to the surface), and 1 prism with large archenteron. Of the 10 halves which remained 3 hours in $\frac{1}{2}$ only 3 survived. Although these had been more exposed to the DNP than those in the

other series, they differentiated completely in conformity with the controls (Text-fig. 8C).

The 12 control vegetal halves developed into more or less ovoid larvae, a typical differentiation of vegetal halves (Text-fig. 9A). A similar development was shown by the treated halves: in $\frac{1}{4}$ for $1\frac{1}{2}$ hours before operation, 8 halves; $1\frac{1}{2}$ hours after operation, 7 halves (Text-fig. 9B); 3 hours before operation, 4



TEXT-FIG. 9. γ-DNP. Vegetal halves. A, controls. B, $\frac{1}{4}$-saturated solution for $1\frac{1}{2}$ hours. C, $\frac{1}{2}$-saturated solution for $1\frac{1}{2}$ hours. (Exp. 50 : 38.)

halves; and in $\frac{1}{2}$ for $1\frac{1}{2}$ hours after operation, 6 halves (Text-fig. 9C). Perhaps a faint deviation in a vegetal direction can be said to exist, but there is nothing like the strong vegetalizing effect in many animal halves. In $\frac{1}{2}$ after 3 hours the vegetal halves were reduced or died.

Some other experimental series gave similarly varied results. These may be briefly recorded. In $\frac{1}{4}$ solution for 2 hours there was the uniform result of 6 plutei, whereas in another series (with another batch of eggs) 4 hours had no effect at all. With the stronger solution ($\frac{1}{2}$) the same eggs that had given 6 plutei formed only 3 plutei out of 6 halves, and the double time, 4 hours, resulted in only 3 plutei out of 7 halves. With another batch of eggs 3 hours in $\frac{1}{2}$ had no effect on 5 halves, whereas 3 hours in the weaker solution ($\frac{1}{4}$) caused 1 out of 9 to begin gastrulation.

α-DNP

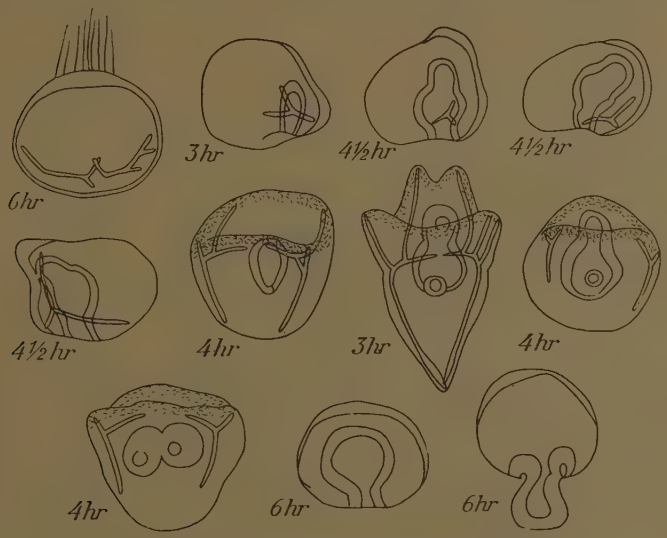
The results were similar in α- and β-DNP. α-DNP was always used in a $\frac{1}{10}$-saturated solution. The results of treating animal halves were as follows. Only the surviving halves are recorded. The different series are separated by dashes.

2 hours: 10 blastulae.—3 blastulae, 2 plutei.—1 blastula, 2 plutei.—9 blastulae, 1 pluteus.—5 blastulae.—10 blastulae.

3 hours: 3 blastulae, 1 gastrula with skeleton, 1 pluteus.—6 blastulae, 2 gastrulae with and 2 without skeleton.

4 hours: 7 blastulae.—6 blastulae, 2 gastrulae without, 2 gastrulae with skeleton.—4 blastulae.—10 blastulae.—6 blastulae, 2 prism larvae, 1 pluteus.—10 blastulae, 1 gastrula, 3 prism or ovoid larvae.

6 hours: 2 blastulae, 1 blastula with skeleton, 1 gastrula.—8 blastulae, 1 prism larva, 3 plutei.—7 gastrulae, 1 gastrula with skeleton, 1 partial exogastrula.



TEXT-FIG. 10. β-DNP, $\frac{1}{10}$-saturated solution.

Some of the vegetalized larvae appear in Text-fig. 10. In one case (left in lower row) the invagination started at two points simultaneously but the archenterons fused in their upper parts. A similar doubling, although with two separate archenterons, occurred also in a series with β-DNP.

β-DNP

A saturated solution was diluted to $\frac{1}{4}$. The results of treating animal halves were as follows.

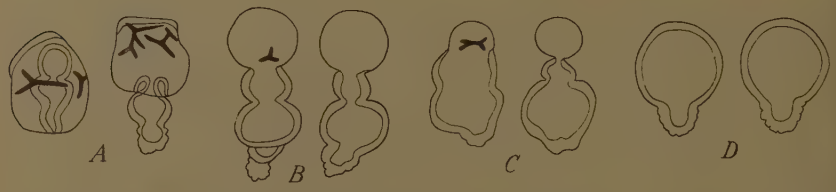
2 hours: 4 blastulae, 2 gastrulae with skeleton.—10 blastulae.—4 blastulae, 2 gastrulae with skeleton.

3 hours: 3 blastulae.

4 hours: 4 blastulae.—1 gastrula with skeleton, 2 plutei.—3 blastulae.—7 blastulae, 3 gastrulae with skeleton.—9 blastulae, 1 incipient gastrulation.—2 blastulae.

CONCLUSIONS

α-, β-, and γ-DNP have the power to vegetalize animal halves in a way resembling the action of lithium ions. There are, however, some remarkable differences. We may find a slight effect resulting in an incipient gastrulation or in skeleton formation in a blastula, but also a much stronger vegetalization, from typical plutei to ovoid larvae of vegetal type and even to exogastrulae with enlarged endodermal region. The effect is, however, very variable and impossible to predict, whereas with lithium treatment the effect is often rather uniform within certain limits, when eggs from one female are treated in an identical



TEXT-FIG. 11. Vegetal halves of *Psammechinus miliaris*. A, the two most common types among the control halves. B–D, the typical forms after treatment by respectively 1·2%, 1·9%, and 2·5% Li-solution.

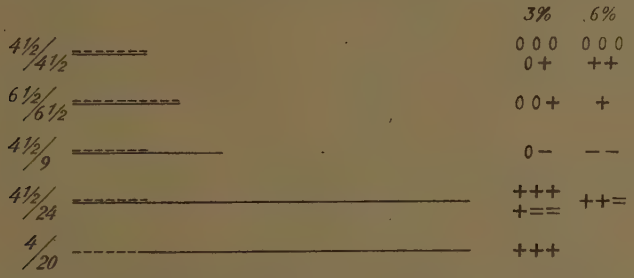
manner. The DNP may affect one or two halves in a series but, particularly in stronger solutions, leave all the other ones unchanged. It was very rarely that all the halves in a series were vegetalized in a similar manner. It was particularly striking that several halves could show considerable vegetalization in a series immersed in a low concentration or for a short time, when stronger solutions or more prolonged treatment had no effect on eggs from the same batch.

It is also interesting to observe that vegetalization seldom occurred in whole eggs, or in vegetal halves, yet the latter showed a higher mortality than animal halves in higher concentrations or with a long treatment. This lack of a gradual increase in the vegetalizing effect of DNP on vegetal halves is in striking contrast to the action of lithium. Text-fig. 11A shows the two types which were dominant among the control vegetal halves as well as the differentiation of vegetal halves treated with 1·2, 1·9, and 2·5 per cent. Li-solution (B–D). The gradual increase of the endoderm to complete endodermization in 2·5 per cent. (D) should be compared with the negative results illustrated in Text-figs. 2 and 9; and the fact that stronger solutions or prolonged time were lethal should also be taken into account.

SIMULTANEOUS ACTION OF α-DINITROPHENOL AND LITHIUM

Motomura (1947) reports that the vegetalizing effect of lithium ions on the development of sea-urchin eggs is cancelled by α-DNP when added to sea-water containing lithium. Motomura further states that the mode of action of α-DNP is distinguished from the animalizing effect of NaSCN (Lindahl, 1936) or that of SO_4-free sea-water (Lindahl, 1935, 1936) by the fact that α-DNP does not lead to animalization when used alone, and that it insensitizes the eggs to lithium by inhibiting the cleavage.

The statement by Motomura that α-DNP has an 'animalizing' effect is astonishing in the light of our results concerning animal halves. That Motomura did not obtain any results when using α-DNP alone on whole eggs is not surprising as,



TEXT-FIG. 12. Diagram of experiments with combined treatment of α-DNP and lithium chloride. The numerator and the broken lines indicate the duration of DNP-treatment, the denominator and the full lines the times in 3% or 6% Li-solution. O, neither Li alone nor DNP+Li had any effect. =, equal vegetalization in Li and DNP+Li. +, stronger vegetalization in presence of DNP than in Li alone. −, lesser degree of vegetalization by DNP+Li than by Li alone. The three experiments of 4/20 were performed with α-, β-, and γ-DNP respectively.

according to the above experimental series, whole eggs are considerably less open to the action of DNP than animal halves, although vegetalization in some cases may be achieved (Text-fig. 5). As our results were so contradictory (Motomura claiming an 'animalizing' effect and I a vegetalizing effect), it was desirable to treat an extensive series with lithium combined with DNP.

It should be mentioned that Lindahl (1940) has found that 4·6-dinitro-*o*-cresol increases the action of lithium.

The experiments were made on many batches of eggs, but the eggs from any one batch were used for several concentrations and periods of treatment. The lithium sea-water contained 3 or 6 per cent. of a 3·5 per cent. solution of LiCl. The α-DNP was used in a $\frac{1}{10}$-saturated solution in sea-water. Only whole eggs were treated. The durations in α-DNP were 4, $4\frac{1}{2}$, and $6\frac{1}{2}$ hours. The eggs did not survive a longer immersion. The lithium treatment varied between 4 hours and 24 hours. As shown in Text-fig. 12, the eggs were as a rule first put in

a solution containing both DNP and lithium and then after $4\frac{1}{2}$ or $6\frac{1}{2}$ hours they were transferred to sea-water, or they were, after $4\frac{1}{2}$ hours, transferred to lithium sea-water of the same concentration for continued treatment for a further $4\frac{1}{2}$ hours or 20 hours. In each experiment the following series were run simultaneously with eggs from the same batch: (1) Controls in normal sea-water. (2) A series in α-DNP only. It can be said now that no deviation from the normal development could be detected in these. (3) A series in 3 or 6 per cent. lithium solution. (4) A series in a combined solution of $\frac{1}{10}$-saturated α-DNP in 3 or 6 per cent. lithium. The results are summarized in Text-fig. 12 and some of the

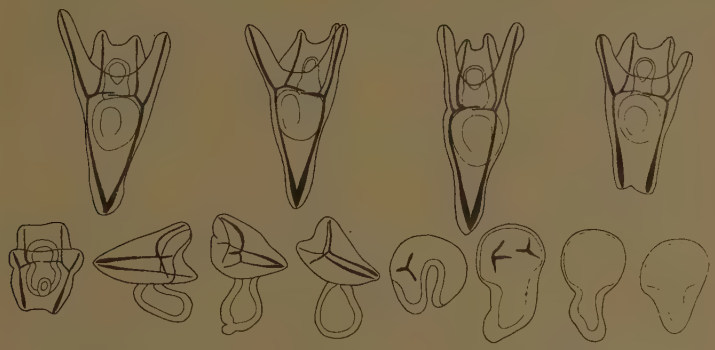
TEXT-FIG. 13. Upper row, Li 6% for $4\frac{1}{2}$ hours. Lower row, α-DNP + Li 6% for $4\frac{1}{2}$ hours.

series are illustrated in Text-figs. 13–17. In Text-fig. 12, 0 means that neither the Li- nor the α-DNP + Li-treatment had any effect; = indicates an equal vegetalization in both kinds of solutions; + means that the vegetalization was stronger in presence of α-DNP than in the Li-solution; whereas − represents a lesser degree of vegetalization by α-DNP + Li than in Li alone.

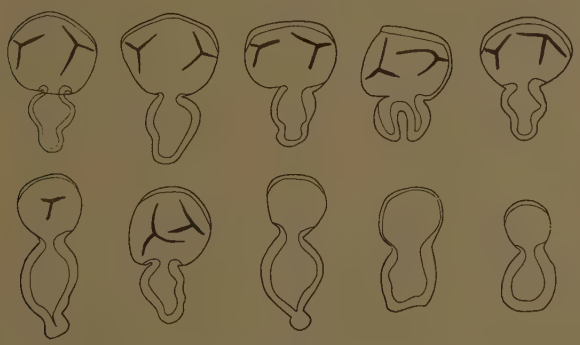
The last experiment in Text-fig. 12, 4/20, differs in two respects from the others. The eggs were first placed in sea-water containing lithium after 4 hours in DNP. Nevertheless the result was positive. Only one of these three series was treated with α-DNP ($\frac{1}{10}$-saturated); the other two were treated with $\frac{1}{4}$-saturated β-DNP and $\frac{1}{2}$-saturated γ-DNP respectively. It is interesting to note that the three substances gave exactly similar results. The Li-controls were exogastrulae with the vegetal tip of the archenteron invaginated and with a fairly well-developed skeleton. In the DNP series the endodermization was more pronounced and the skeleton poorly developed or missing.

$4\frac{1}{2}$ hours as well as $6\frac{1}{2}$ hours was too short a time to give any Li-effect (Text-fig. 12). The larvae treated with Li-solution only form typical plutei (Text-figs. 13, 14). In 9 of the 14 series the eggs in α-DNP + Li also showed no influence, but in 5 series there was a more or less marked vegetalization in many of the

larvae. In Text-figs. 13–17 the different types are shown in approximately the same proportions as they occurred in the cultures. We learn from Text-fig. 13, illustrating one of the 3 positive cases from the series immersed for $4\frac{1}{2}$ hours, that some developed as typical or somewhat atypical plutei when others had become so strongly vegetalized as to form exogastrulae without skeleton. We here observe the same varied effect of the α-DNP as in the experiments with



TEXT-FIG. 14. Upper row, Li 3% for $6\frac{1}{2}$ hours. Lower row, α-DNP + Li 3% for $6\frac{1}{2}$ hours.

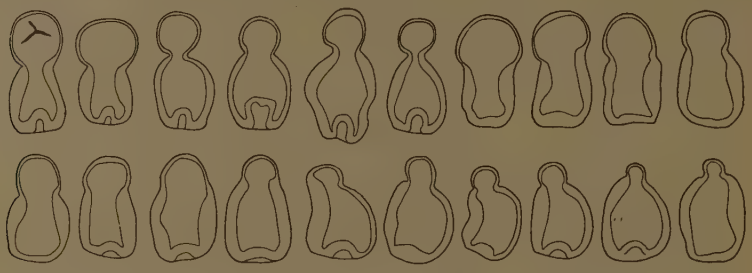


TEXT-FIG. 15. Upper row, Li 3% for 24 hours. Lower row, α-DNP for $4\frac{1}{2}$ hours + Li 3% for 24 hours.

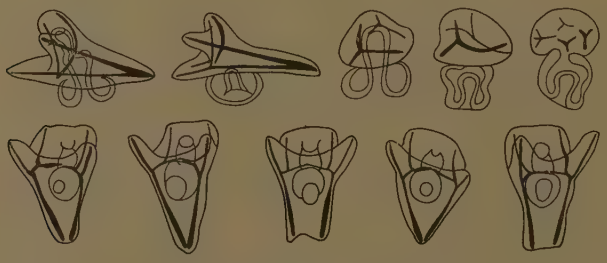
animal halves. The two positive series immersed for $6\frac{1}{2}$ hours were nearly identical with those immersed for $4\frac{1}{2}$ hours (Text-fig. 14). When the larvae had been exposed to lithium for 20–24 hours the vegetalization was pronounced and uniform and resulted in all series in exogastrulae of different types (Text-figs. 15, 16). The degree of vegetalization was identical in the Li-culture and in the corresponding α-DNP + Li-cultures, but only in 3 of the 12 series (Text-fig. 12, $4\frac{1}{2}$/24). In the other 9 series the endodermization had gone further when α-DNP was

present, as is shown for two series in Text-figs. 15 and 16. It is striking that in these series the vegetalization in the α-DNP + Li solution is much more uniform than in the above-mentioned series with shorter Li-treatment—cf. Text-figs. 15, 16 and also 13 and 14. The same holds also for the other series not illustrated here.

The experiments with a duration of $4\frac{1}{2}$ hours in α-DNP + Li followed by another $4\frac{1}{2}$ hours in Li ($4\frac{1}{2}$/9) show peculiar exceptions to the above results



TEXT-FIG. 16. Upper row, Li 3% for 24 hours. Lower row, α-DNP for $4\frac{1}{2}$ hours + Li 3% for 24 hours.

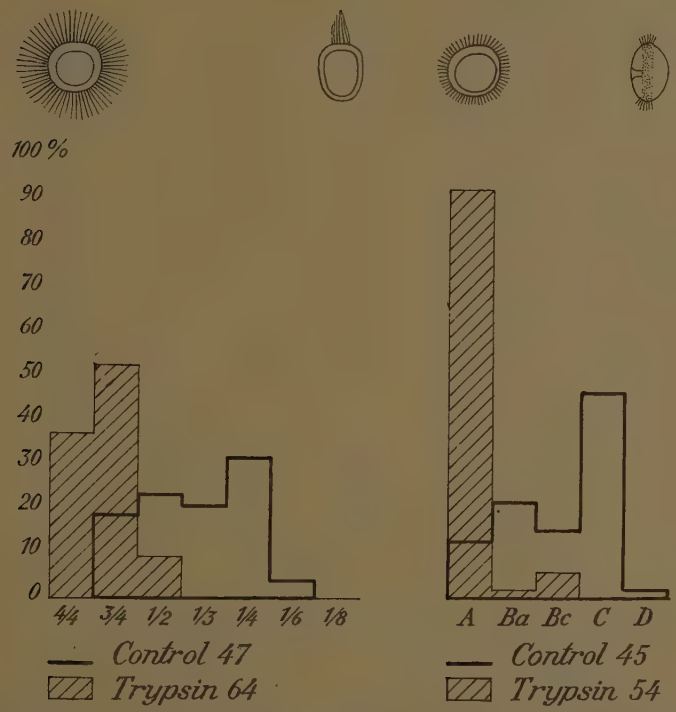


TEXT-FIG. 17. Upper row, Li 6% for 9 hours. Lower row, α-DNP for $4\frac{1}{2}$ hours + Li 6% for 9 hours.

(Text-fig. 12). In 1 out of 4 cases neither solution had any influence. In the other 3 series the Li-cultures were more or less vegetalized, whereas the α-DNP + Li-larvae differentiated in a typical way (Text-fig. 17). It is hard to explain this result, which is contrary to those obtained both with shorter and longer Li-treatment. In 1 of the 3 cases the difference was very slight. The cases are therefore so few that the question arises whether the appearance of the minus cases here is incidental, or due to the particular relation between the times of action of the two solutions.

When one compares these results with those of Motomura, the following is revealed. Motomura treated eggs after fertilization with α-DNP and LiCl simultaneously for 6 hours. During this period the cleavage was at a standstill. They were then transferred to sea-water. This very closely resembles our experiment

$6\frac{1}{2}/6\frac{1}{2}$ (Text-fig. 12). It is not surprising that Motomura found no vegetalization (this lack of effect he calls animalization) as the development is arrested during the treatment and no LiCl-solution is used during the Li-sensitive period, namely during cleavage and blastula formation. Nor did I find any action of the Li-ions



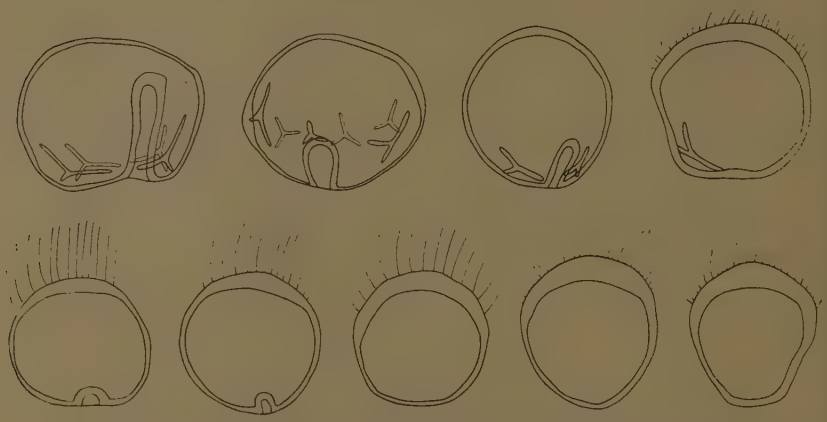
TEXT-FIG. 18. Animalizing effect of trypsin on animal halves. The columns indicate the percentage of different types. Columns with thick lines: control halves. Hatched columns: treated halves. Left row: frequency of the types 4/4 to 1/8 on the first day after operation (4/4 is the most animal type, covered all over with long stiff cilia; 1/8 has a typical apical tuft). Right row: the frequency of the types A to D the following day (A is animal type; D is most vegetal type, with ciliated band and stomodaeum).

alone under similar circumstances ($4\frac{1}{2}/4\frac{1}{2}$ and $6\frac{1}{2}/6\frac{1}{2}$, Text-figs. 12–14), but nevertheless the two substances combined were active (Text-figs. 12–14), a result contradictory to that of Motomura.

ANIMALIZATION BY TRYPSIN AND FICIN

It has previously been observed that the proteolytic enzymes trypsin and chymotrypsin exert a marked animalizing influence on isolated animal halves

(Hörstadius, 1949). The same experiment has now been repeated (Text-figs. 18–20). The halves in trypsin (0·1 and 0·05 per cent.) show more decidedly animal types (see p. 328) than the control halves. Some even had the whole surface covered with long, stiff cilia (Text-fig. 18, 4/4), which otherwise never occurs in isolated animal halves, but has been observed in the isolated most animal quarter (an_1-ring) (Hörstadius, 1935).



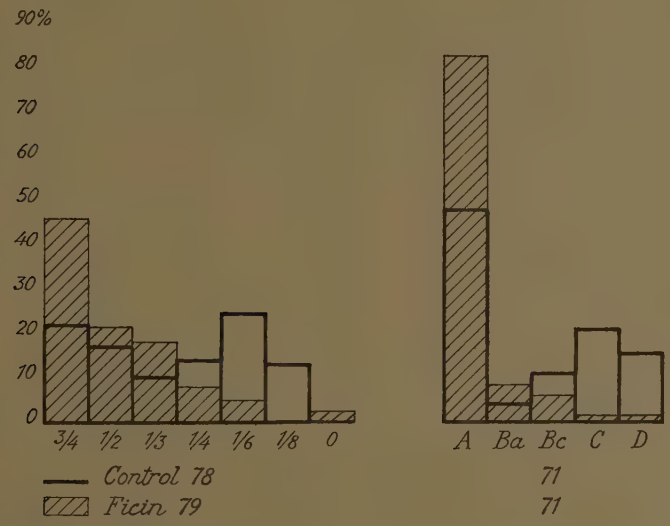
TEXT-FIG. 19. Intact eggs treated by trypsin, after fertilization, for about 20 hours. Upper row 0·05%, lower row 0·1%.

TEXT-FIG. 20. Upper row, vegetal halves treated with 0·05% trypsin. Lower row, control vegetal halves.

Whole eggs and vegetal halves are also open to the action of trypsin. Text-fig. 19 shows a number of larvae from whole eggs with irregular and poor development of the skeleton and a more or less reduced archenteron. In one no invagination has taken place although a spicule has been formed. Three are completely ectodermized, resembling huge animal halves, in an early stage with enlarged apical tuft.

The vegetal halves treated with trypsin (Text-fig. 20, upper row) have a markedly smaller archenteron than the control halves (lower row), and one of them has a thick apical wall.

The proteolytic plant enzyme ficin also had an animalizing effect on isolated animal halves (Text-fig. 21). Whole larvae did not, however, show any change of differentiation.



TEXT-FIG. 21. Animalizing effect of ficin on animal halves. Cf. Text-fig. 18.

DISCUSSION

It was mentioned in the introduction that the animal and the vegetal trends of development in the sea-urchin egg are considered as two opposite gradients representing different types of metabolism. From studies of the respiration in normal and Li-treated eggs Lindahl (1936) has arrived at the conclusion that the animal type is characterized by carbohydrate combustion. This hypothesis has received additional support from the fact that several substances related to carbohydrate metabolism have been found to cause an animalization of isolated animal halves, namely sodium pyruvate (Hörstadius & Strömberg, 1940), propanediol phosphate, phosphogluconic acid, and lactate (Hörstadius & Gustafson, 1947). The same result has also been achieved with intact eggs through treatment with pyruvate (Arosio *et al.*, 1949). Ectodermal development, i.e. the animal metabolic type, favours the development and activity of mitochondria including protein synthesis (Gustafson & Hasselberg, 1951; Gustafson & Lenicque, 1952; Gustafson, 1952).

Li-ions cause vegetalization (Herbst, 1892). In extreme cases they may convert the whole egg to endoderm. Lack of SO_4-ions results in an animalization (Herbst,

1897, 1904) because of an inhibition of the vegetal processes. According to Lindahl (1936), this is due to toxic waste products of aromatic amino acids which normally, in presence of SO_4-ions, are detoxicated by phenolsulphatases. The endomesodermal development, i.e. the vegetal metabolic type, may therefore be characterized by a protein combustion and a sulphate esterification.

It is known that DNP prevents the use of energy provided by respiration and glycolysis, and it has been suggested that it does so by inhibiting the formation of high-energy phosphate bonds (Lardy & Elvehjem, 1945; McElroy, 1947). This hypothesis was supported by Loomis & Lipmann (1948), who showed that low concentrations of DNP reversibly uncouple the phosphorylation associated with the oxidation of glutamate. The depression of oxidative phosphorylation by DNP was confirmed by Judah & Williams-Ashman (1950) using a number of substrates.

DNP has in this paper been shown to cause a strong vegetalization in many cases, transforming animal halves to plutei, even of vegetal type. This implies a morphological counterpart to the action of lithium on animal halves (von Ubisch, 1925; Hörstadius, 1936). The primary point of attack of lithium in the metabolism has long been unknown. The decrease of respiration in the presence of lithium is important. In normal development there are three periods of increasing respiration, the curve showing the shape of an S, a straight line, and an exponential rise. Gray (1927) and Lindahl (1936, 1939) furthermore discern a constant and an increasing part of the respiration. In the first period lithium probably inhibits the increasing but not the constant part; in the following periods it probably also inhibits the constant part, acting in another way (Lindahl, 1939). The vegetalizing effect of lithium is strengthened by lowering respiration through $CO + O_2$, KCN, or partial anaerobiosis (Runnström, 1928*b*, 1933; Lindahl, 1936, 1940). The importance of respiration for the animal trend of development is illustrated by the fact that animalizing agents such as SCN-, I-, and Br-ions are only efficient in presence of O_2 (Lindahl, 1936), and that pyocyanine intensifies their effect (Runnström & Thörnblom, 1938). Lithium is also counteracted by K-ions, with regard to both morphology (Runnström, 1928) and respiration (Lindahl, 1936). Li^+ may competitively displace K^+ from an enzyme (Lindahl, 1936). It has been suggested that K^+ is essential for the phosphate transfer from 2-phospho-pyruvate to ADP (Kachmar & Boyer, 1951). It has actually been shown that an addition of lithium ions to a culture of sea-urchin eggs causes an accumulation of inorganic pyrophosphate during cleavage, i.e. during the period when Li^+ promotes vegetalization (Lindahl & Kiessling, 1951). Lithium evidently, therefore, brings about abnormalities in the phosphate metabolism.

Glutamine causes animalization in animal halves (Hörstadius & Strömberg, 1940; Hörstadius, unpublished). Gustafson & Hasselberg (1951) found that Li treatment during intermediate cleavage stages strongly retards the normal increment of glutaminase activity at the onset of gastrulation. They point out

that the energy released in carbohydrate breakdown is transferred to phosphate bonds which is in accordance with the increase of ATP-turnover at the mesenchyme-blastula stage. The phosphate bond energy would be used *inter alia* for glutamine synthesis which is known to consume ATP (for references see Gustafson & Hasselberg, 1951). This gives us another case where lithium may interfere with phosphorylation, inhibiting a synthesis, and thereby causing a vegetalization.

We have seen that several investigators have brought forward evidence supporting the idea that lithium interferes with phosphorylation, and that Lindahl & Kiessling (1951) have proved a real disturbance of phosphate metabolism by lithium. Similar morphogenetic effects to those of lithium may be achieved by DNP, which is known to inhibit the energy transfer although its real point of attack in the cycle seems to be still obscure. This result gives additional support to the results and hypotheses concerning the action of lithium.

It is of great interest in this connexion that a strong vegetalization including exogastrulation has been obtained by sodium azide (Child, 1948, 1953) which attacks the cytochrome system.

The action of DNP is peculiar in several respects. Its effect may be as strong as that of lithium, transforming even animal halves to plutei of vegetal type. On the other hand, its action is very selective, many larvae in an experiment being altogether unaffected. Furthermore, entire larvae only rarely showed any vegetalization, and vegetal halves either developed like the controls or they disintegrated. On the contrary, various degrees of vegetalization are obtained when vegetal halves are treated with lithium (Text-fig. 11).

Combination of DNP and Li often gave a stronger vegetalization than either of these agents alone. In those series (Text-figs. 12–17) in which whole eggs were treated, the DNP alone had no effect. In some series with DNP + Li the larvae did not deviate from the controls (0 in Text-fig. 12). In 3 series the degree of vegetalization was the same in DNP + Li as in Li alone (= in Text-fig. 12). In the majority of cases where differences between the two existed the larvae in DNP + Li were more vegetalized than the pure Li-larvae (+ in Text-fig. 12, Text-figs. 13–16). Only 3 series gave the opposite result (− in Text-fig. 12, Text-fig. 17). Although the comparatively short early treatment with DNP alone is negative, it evidently prepares the ground for, or strengthens, the action of lithium. This perhaps is another indication that both substances are interfering with the same kind of processes.

The statement that trypsin and chymotrypsin exert an animalizing influence on isolated animal halves (Hörstadius, 1949) has now been confirmed and a strong effect also demonstrated on intact eggs (Text-fig. 19). Moore (1952 *a*, *b*) also claims the same result on whole eggs of *Strongylocentrotus droebachiensis* when exposed to trypsin before fertilization. Subsequent treatment by lithium neutralized the effect. But Moore's photographs give the impression more of inhibited gastrulation than of real animalization.

The statement that the plant enzyme ficin also has an animalizing action on animal halves (Text-fig. 21) invites the question whether all kinds of proteolytic enzymes have a similar effect. Further investigations are needed.

It is not easy to imagine how the proteolytic enzymes work in this case. A complete permeation so that molecules interfere with protein metabolism in the interior is not very likely. Some authors have found that the hyaline membrane is digested by trypsin but cleavage and gastrulation may proceed, whereas other authors claim that the hyaline layer is more or less resistant. The result evidently depends on differences in experimental conditions (literature in Bohus Jensen, 1953). One assumption to explain the animalizing action might be that trypsin changes the permeability of the surface layer so that substances may pass in or out in an abnormal way. Another possible explanation is that the enzyme attacks a superficial substance which is of special importance for the vegetal trend of development. A third explanation is that some proteolytic degradation products from the surface may penetrate to the interior.

SUMMARY

1. α-, β-, and γ-dinitrophenol have the power to vegetalize sea-urchin eggs, particularly animal halves, in a manner resembling the action of lithium ions, but with some differences. The effect is very varied, often only a few of the treated eggs or animal halves becoming vegetalized. Vegetal halves either developed like the controls or they disintegrated; whereas different concentrations of LiCl caused different degrees of vegetalization of vegetal halves.

2. Combination of DNP and lithium often causes a stronger vegetalization than either of these agents alone.

3. The possibility that lithium and DNP attack metabolism at the same point is discussed.

4. Trypsin causes a strong animalization of whole larvae as well as of animal and vegetal halves. An animalization of animal halves was achieved also with ficin.

ACKNOWLEDGEMENTS

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On this occasion I want to acknowledge with gratitude the many inspiring suggestions which Professor John Runnström has given me with regard to my work on determination and metabolism in the sea-urchin egg. I am also indebted to my colleague Professor P. E. Lindahl for valuable discussions.

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Studies on the Mechanism of Meristic Segmentation

I. The Dimensions of Somites

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INTRODUCTION

THE two major aspects of embryonic development are the appearance of differentiated tissues and of organized structures. Most recent investigations have been directed mainly towards the first of these two categories, and have attempted to elucidate the changes in the character of the living substance in terms such as the reactions between organizers and competent tissues, determination by means of gradients or 'organ-forming substances', the influence of genetic factors, and the like. Very much less effort has been devoted to the study of the changes of form undergone by the developing tissues. These geometrical changes must, of course, be brought about by the operation of physical forces, and embryonic development offers a whole range of biophysical problems about which our ignorance remains profound.

This paper reports a study directed towards this general class of biophysical problem. In most embryonic changes of structure both the initial form and the later ones are geometrically complex and difficult to describe in reasonably precise terms. Among the exceptions to this, one instance is the fundamentally important process of the metameric segmentation of the vertebrate dorsal axis. In this a simple sheet of mesoderm—which in blastodermic embryos is nearly plane, while in forms such as the Amphibia is part of the surface of a sphere—becomes segmented into two series of roughly rectangular blocks lying one on each side of the dorsal axis. It would be easy to suggest, and put into mathematical formulation, a number of different types of process which might account for such a segmentation. For instance, phenomena related to the Liesegang's rings might be involved. Or the segmentation might depend on some periodicity which arose from the interaction of two or more chemical processes; Waddington (1940) has referred in this connexion to the system of simultaneous differential equations commonly known in biology as the Volterra equations

$$\frac{\partial x}{\partial t} = ax - bxy, \qquad \frac{\partial y}{\partial t} = cy - dxy$$

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which can be taken to refer to the formation of two substances X and Y, such that each is autocatalytic and tends to destroy the other. If the formation of an inter-somitic groove depended on the concentration of X rising above or falling below some threshold, and if the system entered into operation at the anterior end of the sheet of mesoderm and spread along it towards the posterior so that the periodicity in time became converted into one in space, the formation of the rows of transverse grooves might be explained.

Before much progress can be made in formulating more definite working hypotheses as to the biophysical processes which cause metameric segmentation, it is necessary to obtain further information about the general formal characteristics of the situation. In the types of hypothesis mentioned above, for example, no mention was made of any spatial dimension except that parallel to the anterior-posterior axis, and if this were justified, it would follow that alterations to the medio-lateral extent of the mesoderm sheet, or to its dorso-ventral thickness, would have no effect on the course of the transverse segmentation. It is the purpose of this paper to investigate how far such assumptions are justified. Attention has in the first place been given to the dorso-ventral dimension; the thickness of the mesoderm sheet has been either increased or decreased, and effects of this on the antero-posterior length of the somites assessed.

MATERIALS AND METHODS

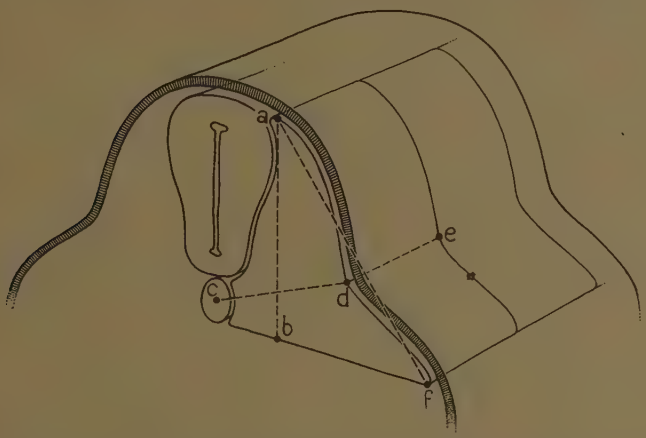
Embryos of *Triturus alpestris* were used. Operations were carried out aseptically, and 0·01 per cent. sodium sulphadiazine (May & Baker) was added to the culture medium, which consisted of one-tenth strength Holtfreter saline, brought to pH 7 by means of phosphate buffer.

The operations were of two main kinds. In the first, an attempt was made to alter the total mass of the embryo during the early stages of gastrulation. As is well known from previous work, relatively harmonious embryos can be formed from early gastrulae which have suffered either a loss of material (e.g. Ruud & Spemann, 1922) or an increase of it (Waddington, 1938). Regulative processes in the altered gastrulae must therefore cause changes in the mass of invaginated mesoderm, and this would presumably mean that the mesoderm sheet would be thicker than normal (or in the opposite case thinner) at the time when it is becoming segmented into somites. If the thickness of the sheet enters into the processes determining the segmentation, there should be a correlation between somite length and thickness in such embryos.

In the second type of operation the thickness of the already formed mesoderm sheet was altered in the late gastrula. The thickness was either reduced by removal of the archenteron roof (presumptive somite material) on one side of the embryo; or increased by the addition of such material under the dorso-lateral ectoderm covering the presumptive somites of an embryo with an intact archenteron roof.

The embryos were allowed to develop to the gill-pouch stage (Harrison 32).

Most of them were then killed in Bouin or in 20 per cent. alcohol; the latter caused perhaps less contraction. Embryos killed in this way were sectioned either transversely or sagittally and stained with Delafield's haematoxylin. Somite dimensions were measured with a micrometer eyepiece. The heights (*ab*) and widths (*cd*) measured on transverse sections are illustrated in Text-fig. 1;



TEXT-FIG. 1. Part of the dorsal axis, to show the dimensions measured. *ab*, height of somite; *cd*, width; both measured on transverse sections. *af*, slant height; *de*, length; measured on whole mount preparations.

it was found that in such series of sections the best estimate of somite length was obtained by counting the number of sections between successive intersomitic septa. In longitudinal sections, only the heights (*ab*) and lengths (*de*) indicated in Text-fig. 1 could be measured; the average value for the three sections nearest the notochord was taken as the best estimate of the true measure.

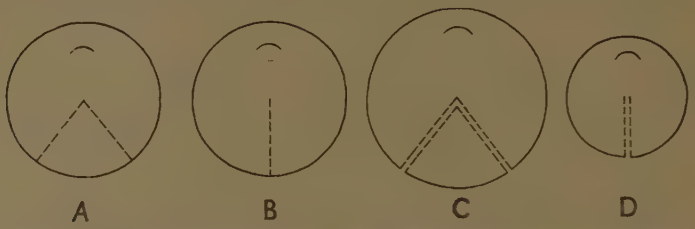
Some embryos were fixed in 5 per cent. formalin, and the complete series of somites dissected from each side, stained with Mayer's haemalum, and prepared as a whole mount. The dimensions measured (*af* and *de*) are also indicated in Text-fig. 1, but the boundaries were not absolutely sharp and this reduced the precision of the measurements.

EXPERIMENTAL RESULTS

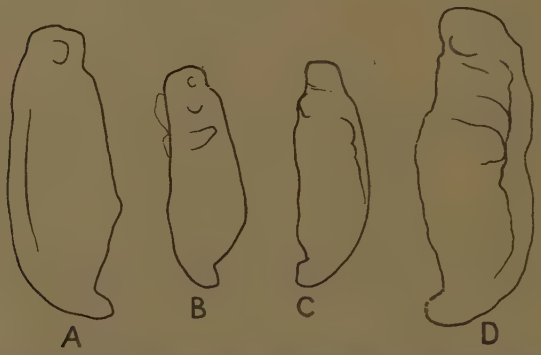
1. *Alterations to the volume of the early gastrula*

Series A. In this preliminary series two methods of altering the volume of the early gastrula were practised. One method was that of Ruud & Spemann (1922); the gastrula was cut in two in the frontal plane by laying on it a thin glass rod, which gradually sank under its own weight and divided off a ventral section of greater or less size; only the dorsal section gastrulates and it usually forms a

fairly well-proportioned though small embryo. In the second method, similar to that used by Waddington (1938) on *Discoglossus*, the reduction in size was produced by removing a large quadrant from the ventral side of a gastrula, the edges of the wound being then carefully brought together and held in place by inserting the egg into a small depression in the operating dish until healing had



TEXT-FIG. 2. Diagram of operation. Young gastrulae seen from the vegetative pole. A, removal of ventral quadrant; B, slitting of ventral side; C, insertion of quadrant from A into ventral slit; D, healing together of ventral side from which a quadrant has been removed.



TEXT-FIG. 3. A, embryo M32*b*–3, with ventral quadrant added to young gastrula. B, M51*b*–3*a*, embryo from the dorsal moiety of a young gastrula cut in two by means of a glass rod; epithelial cysts on head. C, M51*b*–2*a*, embryo from similar dorsal moiety. D, M53*b*–1, with added ventral quadrant.

occurred (Text-fig. 2, A and D). The quadrants reached from the ventral side right up to the animal-vegetative axis, and extended over at least 90° of longitude measured at the equator of the egg. Corresponding increases in volume were produced by making a simple cut in another gastrula from the animal-vegetative axis through to the ventral face, and inserting in this one of the quadrants just described (Text-fig. 2, B and C). Considerable care was needed to obtain satisfactory healing of such large masses, but in favourable cases quite harmonious embryos developed (Text-fig. 3).

Owing to the difficulty of measuring exactly the same dimensions in longi-

tudinal and transverse sections, attention will be confined to those embryos which were sectioned longitudinally. When the dimensions of somites 3–6 were analysed, it was at first found that although both the height and the length were significantly greater in embryos with added tissue than in those with subtracted tissue, there was no significant correlation between these two dimensions. It was then noticed that there was, in several cases, an obvious negative correlation between height and length when the two sides of the same embryo were compared. For instance, in embryo No. 30*b* 1, the figures for a series of somites were (height given first, and dimensions in μ):

	Somite 3	*Somite 4*	*Somite 5*	*Somite 6*
Right side	300 160·5	315 165	300 160·5	300 135
Left side	334 124·5	375 124·5	379 108·5	390 94·5

This situation would appear to be the result of a slight contraction of the somites on one side, leading to an increase in height and decrease in length, while on the opposite side there was a corresponding relaxation. Such a negative correlation within embryos would make it difficult to detect any positive correlation of height with length between embryos. This difficulty can be reduced by averaging the measurements of the two sides, so as to obtain a more valid estimate of the 'resting' dimensions of the somites. This has been done, and the figures are given in Table 1. The correlation coefficient between somite height and length for the whole set of embryos is 0·55, which is not significant ($p = 0{\cdot}05$ for $r = 0{\cdot}632$ with 8 d.f.). Of the embryos included in Table 1, Nos. 30*b* 1 and EG4 had particularly curved dorsal axes, so that the figures for them are likely to be less reliable than the other measurements. If those two are excluded, the correlation of length and height among the others is significant ($r = 0{\cdot}762$, whereas $p = 0{\cdot}05$ for $r = 0{\cdot}707$ with 6 d.f.).

TABLE 1

Large gastrulae	*Average height of somites 3–6*	*Average length of somites 3–6*
30*b* 1*	330·4	132·0
30*b* 2	298·4	149·9
32*b* 1	291·3	184·4
32*b* 3	318·8	139·8
Small gastrulae		
51*b* 1	267·0	147·2
51*b* 2	236·7	104·0
51*b* 3	190·3	106·2
EG 4*	191·7	138·3
EG 9*b*	237·4	130·6

* Axis curved.

Series B. In this series (14 embryos) the alterations of mass were in all cases made by addition or excision of quadrants, which were, however, not as large

as those in Series A, so that the effects on the final size of the embryos were less extreme. The dimensions of somites 3–12 were measured on whole mounts, and again the two sides were averaged. Once more the correlations were positive, and significant, between length and height (dimensions *de* and *af* in Text-fig. 1), over the whole series ($r = 0{\cdot}207$ with 138 d.f., while $p = 0{\cdot}05$ for $r = 0{\cdot}1946$).

2. *Alterations to the thickness of the mesoderm sheet in late gastrulae*

In these experiments the thickness of the mesoderm had been altered on only one side of the embryo, and comparison had to be made between the altered and unaltered side. Any contraction which tended to introduce a negative correlation between height and length could, therefore, not be cancelled out by averaging the two sides. Nevertheless, significant positive correlations were found in these embryos, presumably because at this later stage the power of regulation is less, and the alteration in thickness produced such a pronounced effect on length that it could not be obscured by the consequences of contraction.

In a first series the dimensions of somites 3–12 were measured in whole mounts from a set of 15 embryos in which archenteron roof had been excised from one side and another set in which it had been added to one side. In those which had suffered the loss of tissue the mean of the average dimensions of these somites were as follows (in μ):

Unaltered side: height 411·9 (standard deviation of the distribution 0·929).
length 173·5 (standard deviation 0·280).
Altered side: height 375·7 (s.d. 0·864), length 155·3 (s.d. 0·311).

The correlation of height and length over the measurements as a whole was 0·591 ($p = 0{\cdot}02$, for 13 d.f.).

In a second series 13 embryos were prepared as transverse sections. Measurements were made of width, length, and height (*cd*, *ab*, *de* respectively in Text-fig. 1) of somites 2–5, and an estimate of the cross-sectional area of the somites was calculated as a simple product of the two former. The measurements with their standard deviations are shown in Table 2.

TABLE 2

Mean dimensions in μ

Unaltered side				*Altered side*			
Embryos with archenteron roof excised							
Width	*Height*	*Length*	*Area*	*Width*	*Height*	*Length*	*Area*
224·4 0·616	225·9 0·447	158·7 0·248	503·3 1·245	194·6 0·472	187·3 0·644	150·3 0·332	351·4 1·296
Embryos with archenteron roof added							
222·8 0·259	210·8 0·383	129·2 0·261	472·5 1·179	278·3 0·349	224·8 0·332	147·0 0·153	622·3 0·934

The correlation of length with height or with cross-sectional area on the unaltered side was very small and not significant (– 0·038 and 0·082 respectively, whereas $p = 0{\cdot}05$ for $r = 0{\cdot}5760$ with 10 d.f.); but on the operated side there was a clear correlation between length and height ($r = 0{\cdot}836$, d.f. $= 9$, $p < 0{\cdot}01$), and a smaller, insignificant correlation between length and cross-sectional area ($r = 0{\cdot}482$, d.f. $= 9$, $p > 0{\cdot}1$, whereas $p = 0{\cdot}05$ for $r = 0{\cdot}602$).

DISCUSSION

In spite of a rather large variability in the dimensions of somites even in the unoperated embryos, the experiments described above have given a relatively clear-cut answer. Whenever the height of the somites has been noticeably increased or decreased, there is some evidence of a positive correlation between height and length, and when a considerable number of cases are available, these correlations are statistically significant. It must be concluded that spatial dimensions other than the anterior-posterior one are involved in the causation of the transverse segmentation. That process could not, therefore, be expressed by any system of equations such as the Volterra equations modified only by the addition of terms describing a movement of the reactants from the anterior towards the posterior. If the anterior-posterior axis were taken as the X-axis, then the experimental results show that the process of segmentation must be put in the form of a function which involves not only X but also the dorso-ventral axis (say the Z-axis) and possibly the medio-lateral (Y) axis as well.

There are some general implications of this fact for any theory which involves diffusion. For such theories, the height of the somites could only be of importance if the critical substances have to diffuse through the depth of the mesoderm sheet; but the greater the depth the more would diffusion in that dimension interfere with diffusion along the anterior-posterior axis; from which one would conclude that an increase in the depth of the mesoderm should lead to a shortening of the somites rather than to a lengthening. This makes it appear unlikely that the mechanism does essentially depend on the attainment of a threshold concentration by a diffusing substance.

It should be noted that the height of the somites is only very slightly larger than their length, and it is possible that it is only when these two dimensions are of the same order of magnitude that the former influences the latter.

Turing (1952) has recently shown that periodic structures would be produced from a homogeneous initial state, provided that this contained a number of chemical processes which interacted in certain ways, and that chance perturbations occurred around the initial steady state. After some time a system of spatial periodicities may become well established. It appears rather unsatisfactory to appeal to such an inherently chancy mechanism as this to explain a regular and basic phenomenon of development such as meristic segmentation, though it must be admitted that the shapes of the somites often seem at first to be less definite and regular than they later become, which might point to there being an

element of chance in the first stages of their formation. But in any case, the wavelength of the periodicity which arises under Turing's system is a function solely of the rate constants of the various chemical processes involved, and it is not easy to see how in such a system the influence of depth on length could be explained.

The experimental data, in fact, suggest that one basic factor in the situation may be a tendency of somites to remain always of the normal shape, whatever their size within the rather narrow limits of the experiments. A mechanism dependent on forces acting in the surface of the sheet of mesoderm might seem to be plausible in this connexion; if somite formation could be compared to the breaking up of a jet into drops under the action of surface tension, the relationship between the various dimensions would arise naturally. Several other hypotheses might also be made as to the nature of the forces. One of these will be considered in further detail in a later paper in which the evidence relating to notochord formation (Mookerjee, Deuchar, & Waddington, 1953) will also be brought into the picture.

SUMMARY

1. The dorso-ventral thickness of the mesoderm sheet in Urodele embryos was altered by adding or removing material on the ventral side at the beginning of gastrulation. Similar alterations were made at a later stage of development by direct removal or addition of archenteron roof in late gastrulae.

2. Whenever these operations were successful in changing the height of the somites, there was evidence that their anterior-posterior length was altered in the same sense. In spite of considerable variability in the material, the correlation of somite height and length is significant when a large number of observations are considered together.

3. The bearing of this relation between height and length on the biophysical theories of metameric segmentation is briefly discussed.

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The Distribution of Radioactive Potassium in the Uterus of Pregnant Rats and Guinea-pigs

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INTRODUCTION

RECENT experiments on placental exchange and transfer have made use of the introduction of tracer quantities of a radioactive substance into the maternal circulation to examine its distribution in maternal and foetal tissues and fluids. Such experiments have been performed in different species at varying stages of gestation, using radioactive sodium (Flexner & Roberts, 1939; Flexner & Pohl, 1941; Flexner & Gellhorn, 1942; for other references see Reynolds, 1949, and Marshall, 1952), iron (Vosburgh & Flexner, 1950), and phosphorus (Nielson, 1941; Naeslund, 1951) as tracer substances.

The distribution of injected radioactive potassium ions in the tissue of the body has been examined by a number of investigators (see D'Silva & Neil, 1951; Walker & Wilde, 1952; and Ginsburgh, 1952, for references). It has been found that some cells (e.g. those of liver and kidney) readily exchange their potassium with that of the plasma, whereas others (e.g. red cells and skeletal muscle) exchange their potassium slowly. No experiments have been reported on the distribution of radioactive potassium ions injected in tracer amounts into pregnant animals. In the experiments described below the distribution of radioactive potassium ions injected as ^{42}KCl into non-pregnant and pregnant rats and guinea-pigs in tracer quantities has therefore been studied at different times after injection and at various stages of pregnancy.

MATERIALS AND METHODS

Female albino rats weighing between 170 and 290 g. and guinea-pigs weighing between 1·05 and 1·30 kg. were used. Experiments were made on pregnant rats at times varying from the 11th day of pregnancy until term, and also on non-pregnant animals; examination of the ovaries and of vaginal smears was made to determine the point in the cycle at which the non-pregnant animals had been killed.

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Measurement of radioactivity of tissues

The potassium salt used was irradiated 'Specpure' K_2CO_3, solutions of ^{42}KCl being prepared as indicated in the paper by D'Silva and Neil (1951). The solutions made up for injection were 2 per cent. or 0·2 per cent. W/v KCl in distilled water, the volume injected in experiments of short ($\frac{1}{2}$ min. to 6 hours) duration containing less than 2·1 mg. of K ion.

Each rat was anaesthetized with sodium amytal (30–40 mg. intramuscularly) and the ^{42}KCl solution was injected slowly over a period of about 1 minute into the femoral vein. After the appropriate time from the start of the injection the animal was bled from the carotid artery until dead. The uterus, pregnant or non-pregnant, was removed at the cervix and freed from the connective tissue of the broad ligament. The pregnant uterus was opened and the tissues which were removed were placed in separate beakers. The labyrinthine placenta separated easily from the uterus at the junctional zone; histological examinations in several animals revealed that little or no element known to be of foetal origin remained with the uterine tissue. The right kidney was also removed from every animal as a control.

After weighing, the organs were dissolved in hot concentrated HNO_3, foaming being minimized by the use of anti-foam silicone grease smeared on the stirring rods. The digests were made up to a known volume with water and the radioactivity determined with an M.6 liquid counter (Veall, 1948). The results, and those from a standard solution, were corrected for background radiation, decay since the time of injection, and for the resolving time of the counter. Recovery experiments were also carried out on a number of rats by dissolving the remainder of the carcass in HNO_3 and 'counting' the diluted digest. The mean recovery (10 experiments) was 99·0 per cent. of the total radioactivity injected (range 91·9–101·0 per cent.).

Determination of potassium in tissues

Rats were anaesthetized with sodium amytal and bled from the carotid artery. The tissues were rapidly removed, weighed, and dried overnight at 110° C. The weight of dried tissue was then determined.

The dried and powdered tissue (30–40 mg.) was weighed into a centrifuge tube and ashed for 2 hours in a boiling-water bath with 0·2 ml. of nitric acid, diluted with 0·2 ml. of water. The solution was transferred quantitatively to a graduated flask, diluted with water, and the K content determined by means of a direct reading flame photometer (Barnes, Richardson, Berry, & Hood, 1945).

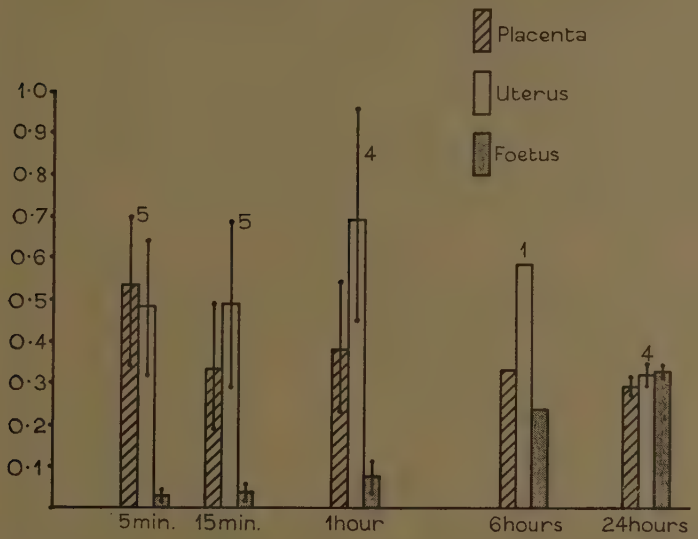
RESULTS

Series 1. Injection of ^{42}KCl into rats at or near term

Aqueous ^{42}KCl containing 1·05 to 2·1 mg. potassium was injected intravenously into pregnant rats 20–21 days after they were mated, and, in separate

experiments, the animals were bled to death 5 and 15 minutes, and 1 hour, after injection. Intraperitoneal injection was used for experiments lasting 6 and 24 hours. One kidney, the uterus, placentae, and foetuses were removed, and their radioactivity determined as described above.

The results for the uterus, placentae, and foetuses are shown in Text-fig. 1,



TEXT-FIG. 1. Activity of uterus, placentae, and foetuses at various times after injection of ^{42}KCl into rats. (Results are expressed as percentage (x) of total radioactivity. x is defined by the expression

$$x = \frac{\text{counts/min./g. tissue}}{\text{total injected counts/min.}} \times 100\, D,$$

where D is a correction factor allowing for decay.) The number of animals in each group is given above the columns. The vertical lines represent the scatter of the observations.

which also shows the scatter in the results for each group of animals, and the number of animals in the group.

The kidney was analysed for radioactivity in each rat and served to show whether the experiments were satisfactory because, from many other experiments performed in this laboratory, it has been found that the behaviour of the kidney in exchanging its intracellular potassium is closely repeatable. Every experiment carried out in each series has been included in this paper.

Five minutes after injection each gramme of kidney tissue held 4·35 per cent. (range 2·35 to 5·88 per cent.) of the total radioactivity. Within the next 10 minutes this figure was reduced by half, and thereafter continued to decrease, until after 6 hours the figure was scarcely greater than that after 24 hours. The radioactivity of the foetal tissues as a whole, on the other hand, gradually increased and was greatest in these experiments after 24 hours.

The results for the kidney and foetus, found in experiments lasting from 5 minutes to 24 hours, are characteristic of those given respectively by tissues which exchange their intracellular potassium rapidly and those which exchange it slowly.

The changes in the amount of radioactivity in the placenta and uterus in similar experiments are clearly not similar to either of the two curves characteristic of fast exchanging or of slow exchanging tissues. In the first few minutes after injection the placenta has a significantly greater activity than after 24 hours ($P < 0{\cdot}01$), but after only 15 minutes the mean value of its activity is not significantly different ($0{\cdot}6 > P > 0{\cdot}5$) from the mean value at 24 hours. There was no significant difference between the mean activity of the uterus after 5 and 15 minutes, or after 15 minutes and 1 hour ($0{\cdot}2 > P > 0{\cdot}1$).

TABLE 1

The activity of tissues in the pregnant rat 5 minutes after intravenous injection of ^{42}KCl

Activity defined as in Text-fig. 1

Rat No.	*Duration of pregnancy in days*	*Foetal length (mm.)*	*Kidney*	*Uterus*	*Placenta*	*Foetus*
55	11	< 6	5·74	0·825	1·13	—
41	13	6	3·41	0·465	0·542	—
35	14	7	6·68	0·406	0·910	0·022*
34	14	8	3·15	0·313	0·391	0·030*
43	14	9	3·33	0·509	0·303	—
44	14	9	2·98	0·501	0·315	—
63	18	9	4·94	0·660	0·545	—
36	14	10	4·32	1·14	0·689	0·015*
37	14	10	5·34	0·757	0·601	—
46	15	10	4·03	0·565	0·322	—
38	15	12	5·48	0·822	0·635	—
39	15	12	5·27	0·693	0·509	—
61	17	17	5·51	0·568	0·350	—
60	17	18	4·01	0·590	0·383	—
Mean	—	—	4·59	0·630	0·545	—

* Includes foetal fluids.

Series 2. Injection of ^{42}KCl *into rats 11–18 days pregnant*

In another series of experiments the distribution of ^{42}K in the uterus, placentae, kidney, and foetuses of rats pregnant for 11 to 18 days was studied 5 minutes after intravenous injection of ^{42}KCl. The amount of K ion injected in each experiment was 0·3 to 1·2 mg., an amount insufficient materially to alter the total amount of K in the extracellular fluid. The results of the experiments are given in Table 1.

It will be seen that the foetal length ranged from less than 6 mm. to 18 mm., and there appears to be a close correspondence between the foetal length and

the shape of the conceptus. The smaller foetuses were found in spheroidal conceptuses. Our observations agree with those of Reynolds (1949) in that there is a change in shape of the conceptus from a spheroid to a cylindrically shaped body at about the 17th day of pregnancy in the rat.

From columns 5 and 6 it appears that with the smallest foetuses (foetal length < 9 mm.) the radioactivity in the uterus was less than that in the placenta. When the foetus was 9–18 mm. long, the reverse was true. This observation was not studied further.

Of the five experiments carried out on pregnant rats at full term (Series 1), the ratio of 'counts' per gramme of uterus to 'counts' per gramme of placenta was greater than 1 in two experiments and less than 1 in three. In only three experiments (Table 1) were measurements made on the activity of foetuses and foetal fluids as the uptake of radioactive material by these tissues in 5 minutes was very small.

In every experiment the activity of kidney tissue was measured and found to be high (Table 1, column 4). The mean values for kidney in the experiments of Series 2 and 4 (of Tables 1 and 3) are not significantly different, but they are lower than the mean value obtained by D'Silva & Neil (1951), in whose experiments the rate at which the radioactive material was injected was slower. The standard deviation was, however, nearly the same (viz. $\pm$ 1·14, $\pm$ 1·34, and $\pm$ 1·12) in the experiments of Series 2, Series 4, and those of D'Silva & Neil, respectively. It is likely, though the point has not been investigated, that the value obtained for the activity of the kidney depends to some extent on the rate at which the radioactive material is injected.

TABLE 2

The activity of tissues of the pregnant guinea-pig 5 minutes after intravenous injection of ^{42}KCl

Activity defined as in Text-fig. 1

Guinea-pig	*Foetal length in mm.*	*Maternal kidney*	*Placentae*	*Foetuses*	*Uterus*
1	100	1·55	0·260	0·017	0·101
3	115	2·04	0·466	0·045	0·130
4	73	4·14	0·343	0·013	0·145
5	72	4·10	0·370	0·017	0·146
Mean	—	2·96	0·360	0·023	0·131
7 (after 1 hour)	105	0·421	0·098	0·032	0·207

Series 3. Injection of ^{42}KCl *into pregnant guinea-pigs*

Pregnant guinea-pigs under Nembutal anaesthesia were slowly injected intravenously with an aqueous solution of ^{42}KCl (0·5 ml. containing 1 mg. K ion). After 5 minutes from the start of the injection the animals were bled and various

tissues taken for assay of their radioactivity. The results are in Table 2. It will be seen that although the percentage of the total radioactivity injected found in each gramme of tissue was lower than for the corresponding tissues of the rat (cf. Table 1), the ratios of the figures for placenta, uterus, and foetus as compared with the kidney were 8·2, 22·6, and 129 for the guinea-pig and 8·2, 9·1, and 137 for the full-term rat. The only noteworthy difference is in the figures for the kidney/uterus ratio in the two species. Apart from this difference the two species behave similarly.

Table 2 includes the results of an experiment in which the radioactivity of the tissues was determined 1 hour after ^{42}KCl was injected intravenously. For the guinea-pig, the ratios of counts/g. kidney to counts/g. placenta, uterus, and foetus were 4·3, 2·0, and 13·2 respectively. The corresponding figures for the full-term rat were 2·3, 1·3, and 11·9, ratios which are of the same order of magnitude as those found for the guinea-pig.

From these experiments, therefore, it appears that the tissues of the pregnant uterus of the guinea-pig behave like those of the rat in response to an injection of ^{42}KCl.

Series 4. The non-pregnant uterus and the non-pregnant horn in the pregnant uterus

A number of experiments were carried out in which ^{42}KCl was injected into non-pregnant rats and the animals were killed 5 minutes afterwards, the radioactivity in the uterus and kidney being determined. The results are given in Table 3.

TABLE 3

The activity of tissues of the non-pregnant rat 5 minutes after intravenous injection of ^{42}KCl

Activity defined as in Text-fig. 1

Rat No.	*Weight of uterus in g.*	*Kidney*	*Uterus*
48	0·257	3·52	0·568
40	0·308	3·33	0·486
42	0·379	3·35	0·780
45	0·566	3·71	0·431
62	0·589	3·60	0·562
54	0·626	5·97	0·988
53	0·686	6·82	0·769
47	0·790	3·94	0·326
Average	0·525	4·28	0·614

It will be seen that the activity of the uterus (Table 3, column 4) is the same 5 minutes after injection in the non-pregnant and in the pregnant uterus (cf. Table 1, column 5 and Text-fig. 1). There is a considerable scatter in the weights of the uteri studied, as well as in their radioactivity per gramme.

As the occasion arose the activity of the non-pregnant horn in an animal in which the foetuses were limited to one horn was estimated. The results are spread over a number of series of experiments and are given in Table 4.

TABLE 4

The activity of the pregnant and non-pregnant horns of the rat uterus at various times after intravenous injection of ^{42}KCl

Activity defined as in Text-fig. 1

Time after injection / *Rat No.* / *Foetal length*	*Weight of pregnant horn in g. and No. of foetuses in parentheses*	*Weight of non-pregnant horn in g.*	*Activity of uterus*	
			Pregnant horn	*Non-pregnant horn*
60 seconds No. 15 42 mm.	0·875 (5)	0·390	1·32	0·755
5 minutes No. 37 10 mm.	0·852 (5)	0·246	0·757	0·585
5 minutes No. 7 37 mm.	2·28 (5)	0·425	0·500	0·710
24 hours No. 22 22 mm.	2·02 (7)	0·460	0·347	0·294

It will be seen that the activity of the pregnant horn is greater in 3 out of the 4 rats than that of the non-pregnant horn, but these differences are probably not significant. The activity of the non-pregnant horn in a unilateral pregnancy is of the same order as that found in the uterus of the non-pregnant animal (cf. Table 3).

Series 5. Distribution of activity within the placenta

In one experiment the peripheral portion of the labyrinthine placenta from a guinea-pig near term was 'counted' separately from the central portion. In three other guinea-pigs, also near term, the labyrinthine placenta was removed and placed in chilled acetone. Sections were cut and mounted, and the tissue scanned by Dr. G. Bourne, using a 'microscope' counter with an aperture 1 mm. in diameter. In no experiment was any significant difference found in the activity of different areas of the labyrinthine placenta.

A few observations were made on the uptake of ^{42}K by the vascular splanchnopleur and amnion of the rat. Fifteen minutes after injection these membranes contained only 0·065 per cent. (per gramme of tissue) and after 1 hour only 0·105 per cent. of the total radioactivity injected. The radioactivity of the amniotic

fluid in the guinea-pig was measured 1 hour after injection and was found to be 0·0069 per cent. of the total radioactivity. These observations are being investigated further.

Series 6. Site of placenta in the horn

Experiments were performed on rats to determine whether any difference could be observed in the activity of the placenta according to its site in the horn. The most cranially placed placentae from each horn, the centrally placed, and the most caudally placed were removed and counted in separate batches (cf. Table 5). No. 2 was more cranially placed than No. 1 and No. 5 more than No. 4, and so on.

TABLE 5

Distribution of radioactivity at different placental sites after intravenous injection of ^{42}KCl

Activity defined as in Text-fig. 1

	Site of placenta	*Placentae*	*Activity %*
(*a*) *Rat killed 30 sec. after injection (foetal length 39 mm.)*	Caudal	Lt. 1 and 2	0·640
		Rt. 1 and 2	
	Central	Lt. 3	0·685
		Rt. 3 and 4	
	Cranial	Lt. 4 and 5	0·630
		Rt. 5 and 6	
			Mean 0·652
(*b*) *Rat killed 60 sec. after injection (foetal length 42 mm.)*	Caudal	Rt. 1 and 2	0·680
	Cranial	Rt. 3, 4, and 5	0·710
			Mean 0·695
(*c*) *Rat killed 15 min. after injection (foetal length 27 mm.)*	Caudal	Rt. 1 and 2	0·317
		Lt. 1 and 2	
	Central	Rt. 3, 4, and 5	0·285
		Lt. 3	
	Cranial	Rt. 6 and 7	0·314
		Lt. 4 and 5	
			Mean 0·305

These results show that placentae in different parts of the horn do not appear to have a significant advantage or disadvantage in regard to the availability of potassium.

A further experiment was performed in which the uterus of a rat, pregnant with six foetuses 38 mm. in length in each horn, was exposed under anaesthetic and the part of the right horn containing the cranial four foetuses was excised. The remaining two placentae on the right side were therefore being supplied only by the right uterine artery. The rat was killed 5 minutes after injection of ^{42}KCl, and the two caudal placentae on each side were 'counted'. The two placentae on the right (operated) side took up 0·713 per cent. and the two on the left

(normal) side 0·646 per cent. per g./min. of the total radioactivity, the difference being not significant.

TABLE 6

Water and potassium contents of the placenta and uterus

	Water content as % of wet tissue		*Potassium content (m. eq. K/Kg. dry tissue)*	
No. of rats	*Placenta*	*Uterus*	*Placenta*	*Uterus*
9 (pregnant)	—	82·6 (80·2–84·5)	—	409 (344–499)
4 (pregnant)	84·7 (84·1–85·3)	—	372 (340–409)	—
9 (non-pregnant)	—	81·3 (78·7–84·3)	—	403 (328–479)

The potassium content of the tissues of the pregnant rat uterus

It will be seen from Table 6 that the mean water and potassium contents of the uterus of pregnant and non-pregnant rats is the same, as is the scatter of the individual observations. In one experiment in which only one horn contained foetuses, each uterine horn was analysed separately. The pregnant horn contained 403 and the non-pregnant horn 407 m. eq. K/Kg. dry tissue and the water content of each was 82·8 per cent. A few determinations were made on the placenta and serve to indicate that this tissue, like the uterus, is rich in potassium.

DISCUSSION

When tracer quantities of radioactive potassium salts are injected intravenously into animals, the radioactivity of the plasma very rapidly declines from its initial high level (Walker & Wilde, 1952; Ginsburg, 1952). This is because the labelled potassium ions are very rapidly distributed throughout the extracellular space and immediately begin to exchange with the intracellular potassium. When the cells of a tissue are in equilibrium with the surrounding extracellular fluid the number of potassium ions entering the cell in unit time is equal to the number of potassium ions leaving the cell in the same time, with the result that the cell suffers no net loss or gain of potassium. When the cell is not in equilibrium with its extracellular fluid, there may be a net loss or gain in intracellular potassium, and this is brought about by the different magnitudes of the outward and inward potassium fluxes. In either of these states labelled potassium ions (^{42}K) can take part equally with the non-radioactive (^{39}K) ions in the movements of potassium into and out of cells. When the flux is large the cell takes up ^{42}K rapidly; when it is small ^{42}K is incorporated into the cell slowly. In either case, the proportion of ^{42}K ions in the total of ions entering the cell is the same as the proportions of the two ions in the extracellular fluid bathing the cell. When equilibrium conditions are reached, the labelled ions entering the cell in unit time form the

same proportion of all the potassium ions entering, as do the labelled ions leaving the cell in comparison with all the potassium ions leaving the cell. Thus there is neither any net change in intracellular potassium nor any change in the radioactivity of the tissue—other than that due to decay of the radioactive material. At equilibrium the relative radioactivities of the tissues are the same as their relative potassium contents.

On the basis of the above argument it is apparent, therefore, that the intracellular potassium of the kidney exchanges rapidly with the extracellular fluid, whereas the foetus, as a whole, exchanges slowly (cf. Text-fig. 1). The placenta fits into neither of these groups. Within 5 minutes of injection it acquires a degree of radioactivity which is not greatly different from the value after 24 hours. If the assumption is made that placental cells behave in the same manner as other cells, the only reasonable explanation of the results obtained seems to be that the cells of the placenta are fast exchangers of potassium and that the blood-flow through the placenta is slow.

D'Silva & Neil (1951) have shown that the relative activities of kidney and liver (both rapidly exchanging tissues) following the administration of ^{42}KCl depend on the route of injection. Thus, after intraperitoneal injection the ratio activity of kidney/activity of liver is, on the average, 1·04, but, after intravenous injection, the ratio is 4·70. This was interpreted in terms of the 'availability' to the two organs of ^{42}K ions following the injection. After intraperitoneal injection the liver is the more favourably situated organ and it is perfused with blood richer in ^{42}K than that which perfuses the kidney. After intravenous injection the kidney is the more favourably placed because much of the blood perfusing the liver has been depleted of its ^{42}K by the tissues of the gut.

A slow blood-flow through a tissue will also make less ^{42}K available for exchange. It is known that the maternal blood-flow through the pregnant uterus of the rabbit (Barcroft & Rothschild, 1932; Barcroft, Herkel, & Hill, 1933) is of the order of 0·3 ml./g. of tissue/minute. In a few experiments on pregnant guinea-pigs and rats we have obtained blood-flows of the same order of magnitude. By comparison, the blood-flow through the kidney is very rapid, so one would expect that if the cells of the kidney and placenta exchanged potassium equally readily, the latter would acquire a smaller proportion of the injected radioactivity than the former in a 'five-minute experiment'.

These experiments give no information about the rate of passage of K ions from mother to foetus, but they indicate that this transport occurs and also that there are cells in the placenta which can readily exchange their potassium with that in the maternal plasma. The labyrinthine placenta of the rat near term consists of trophoblast cells and those of the foetal endothelium, but it is not yet possible to indicate which are the cells that exchange their potassium. It can only be concluded that the potassium exchanges freely with all the placental cells or with some cellular component evenly distributed through the labyrinthine placenta, and that this component might well be part of the placental 'barrier'.

There has been controversy about the nature of the layers of tissue placed between the foetal and maternal blood-streams in the rodent. Mossman (1926, 1937) advanced the theory that the placental barrier of rodents is in many places haemo-endothelial, whereas Hard (1946), as a result of his histochemical investigations, concluded that syncytium must be present as a continuous sheet throughout the labyrinth and that the placenta, at least of the guinea-pig, cannot be haemo-endothelial. Wislocki *et al.* (1946) suggest that, from the point of view of the physiological behaviour of the barrier, it may well make 'relatively little difference what its morphological derivation happens to be'.

SUMMARY

1. The distribution of tracer amounts of ^{42}KCl was studied in the kidney, placenta, uterus, and foetus (*a*) 5 minutes to 24 hours after injection into pregnant rats (20–21 days), and (*b*) 5 minutes after injection into pregnant rats (11–18 days) and guinea-pigs (near term).

2. The changes in activity in the kidney and foetus (per gramme of tissue) indicate that the former exchanges its potassium rapidly and the latter slowly.

3. The activity of the labyrinthine placenta of rats near term falls from 0·53 per cent. (per gramme of tissue) of the total injected radioactivity after 5 minutes to 0·29 per cent. after 24 hours. The activity of the uterus changes similarly and suggests a rapid rate of exchange and a slow blood-flow.

4. The radioactivity of the uterus was not significantly different in the non-pregnant uterus, the non-pregnant horn of the pregnant uterus, and the pregnant horn.

5. The placentae in different parts of the horn do not appear to have a significant advantage or disadvantage in regard to the availability of potassium.

6. The amniotic fluid and vascular splanchnopleur contained only small amounts of radioactivity. The labyrinthine placenta was uniformly active throughout.

7. The potassium content of the labyrinthine placenta, pregnant and non-pregnant uterus, has been determined.

ACKNOWLEDGEMENTS

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Studies on the Development of the Foregut in the Chick Blastoderm

2. The Morphogenetic Movements

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INTRODUCTION

THE first of this series of papers (Bellairs, 1953) outlined the morphological changes occurring in the endoderm of the chick up to the stage of about ten pairs of somites; it also showed that the presumptive foregut area in the primitive streak stage blastoderm lies around the anterior end of the primitive streak, and at a slightly later stage around the head process. The present paper is concerned with the pattern of morphogenetic movements which occur in the endoderm during the early stages of foregut development.

METHOD

The technique involved the use of carbon marks upon the exposed endoderm of blastoderms grown dorsal side downwards in tissue culture by the watchglass method (Waddington, 1932; Bellairs, 1953). During the period of development investigated an expansion of the blastoderm as a whole took place over the plasma clot; the use of external reference points was therefore essential, although even with such aids it was not always possible to tell whether certain displacements of marked cells were in fact merely the expression of a general expansion or were due to a specific morphogenetic migration. Long straight lines of carbon were consequently used in most experiments; they were applied with a fine knife and placed either at right angles to the primitive streak and head process or parallel to them, and extended across the area opaca and on to the clot surface. In some cases splinters of glass were placed on the clot as external reference points. Each blastoderm was drawn to scale and the position of the mark plotted, both before and after incubation. The individual dots and lumps of carbon which composed each line frequently became broken up with the expansion of the blastoderm. A total of fifty-seven blastoderms have been used.

The terms L and *h.p.* have been employed throughout to signify the long or

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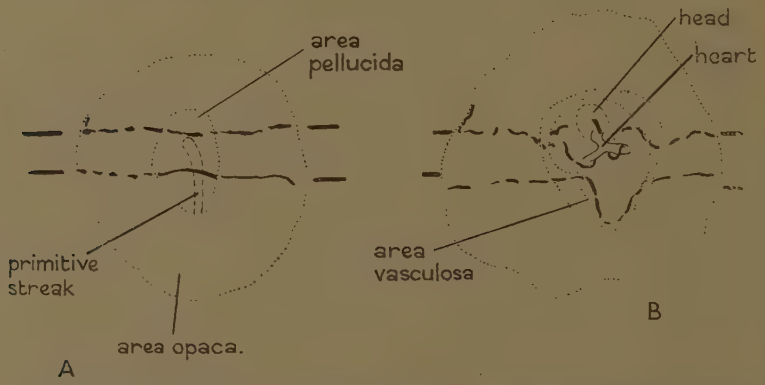
definitive primitive streak stage (as defined by Waddington, 1952, and Abercrombie, 1950) and the head process stage respectively. The following abbreviations are also used in the paper: *p.s.* for primitive streak; *a.p.l.* for area pellucida length.

RESULTS

1. *Marks placed at right angles to the primitive streak or head process*

Specimen No. 1 (L stage; *p.s.* 2·0 mm.; *a.p.l.* 2·6 mm.). Two parallel lines were placed across the blastoderm at right angles to the primitive streak (Text-fig. 1A), the anterior one being immediately in front of and touching the primitive node,



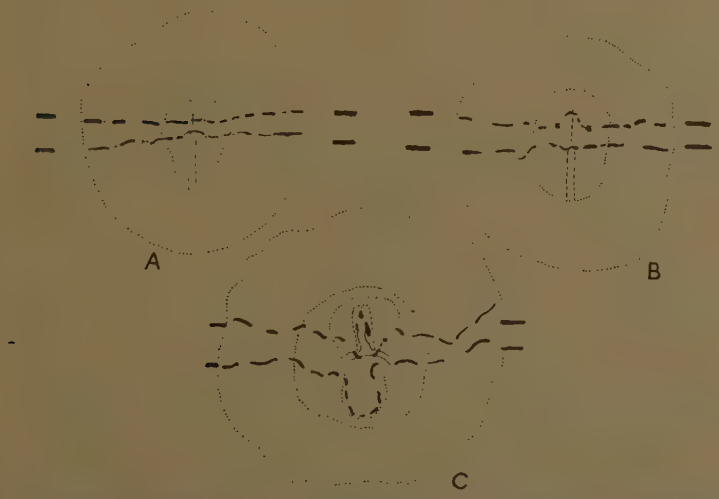
TEXT-FIG. 1. Specimen No. 1. A. Position of carbon marks placed on the endoderm of an L-stage blastoderm. The primitive streak is shown with broken lines; the area pellucida and area opaca are outlined in dots. B. The same marks after 24 hours' incubation. The head is indicated with broken lines and the developing heart as a thin continuous line. The area pellucida, area vasculosa, and area opaca are outlined in dots. The foregut is not shown.

and the posterior one crossing the primitive streak half-way along its length. *After 24 hours* part of the anterior mark had become enclosed in the developing foregut. The carbon which formerly lay just anterior to the node had moved forward slightly and was found in the roof. The parts of the mark which originally lay immediately to either side, however, had moved posteriorly and were adherent to the developing foregut floor. The change in position which the hinder carbon line had undergone showed that an extensive backward migration had occurred along the posterior part of the primitive streak and in the area pellucida endoderm at either side of the midline.

Specimen No. 2 (L stage; *p.s.* 2·2 mm.; *a.p.l.* 2·8 mm.). A line of carbon was placed across the blastoderm at right angles to the primitive streak; it passed just behind the primitive node (Text-fig. 2A). *After 24 hours* a backward movement had again occurred, and had been confined to the area opaca and the regions

lateral to the primitive streak. There appeared to have been no movement of the mark where it crossed the primitive streak.

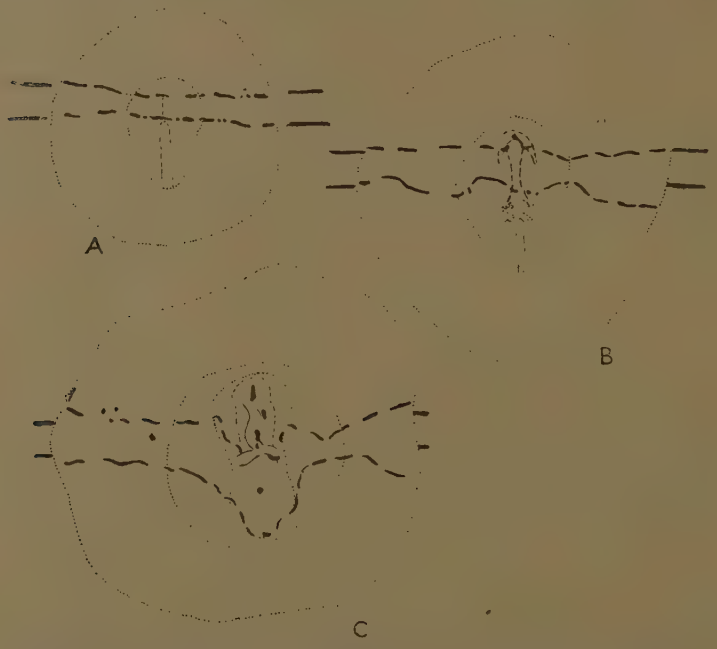
TEXT-FIG. 2. Specimen No. 2. Structures shown as in Text-fig. 1. A. Position of a carbon mark placed on the endoderm of an L-stage blastoderm. B. The same mark after 24 hours.



TEXT-FIG. 3. Specimen No. 3. Structures shown as in Text-fig. 1. A. Position of carbon marks placed on the endoderm of an *h.p.* stage blastoderm. B. The same marks after 5 hours. C. The same marks after 24 hours.

Specimen No. 3 (*h.p.* stage; *p.s.* 2·0 mm.; *h.p.* 0·6 mm.; *a.p.l.* 3·1 mm.). Two lines of carbon were placed across the head process and at right angles to it, one half-way along its length, and the other just touching the tip of the primitive node (Text-fig. 3A). *After 5 hours* a slight forward movement had taken place in the

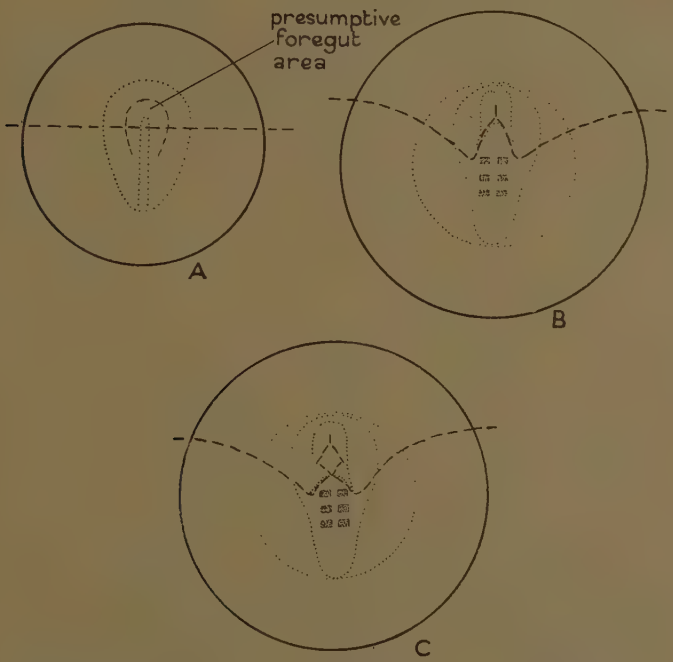
region of the anterior line, but there was no evidence of any displacement of the hinder mark (Text-fig. 3B). *After 24 hours* some of the material of the anterior mark had become enclosed in the foregut (Text-fig. 3C). The more anterior part of it lay in the roof and had undergone a slight forward movement. The more posterior part was adherent to the foregut floor and had moved backwards. In the extra-embryonic area pellucida on either side of the head process the lines had been displaced backwards. The posterior mark showed that an extensive migration had taken place along the primitive streak and, to a lesser degree, in the regions on either side.



TEXT-FIG. 4. Specimen No. 4. Structures shown as in Text-fig. 1. A. Position of carbon marks placed on the endoderm of an *h.p.* stage blastoderm. B. The same marks after 4 hours. C. The same marks after 18 hours.

Specimen No. 4. (*h.p.* stage; *p.s.* 1·8 mm.; *h.p.* 0·9 mm.; *a.p.l.* 2·8 mm.). Two transverse marks were placed at right angles to the primitive streak, the anterior one being immediately anterior to the head process, whilst the posterior one was just in front of the node (Text-fig. 4A). *After 4 hours* part of the anterior mark had become enclosed in the developing foregut and appeared to have migrated slightly forward (Text-fig. 4B). *After 18 hours* (Text-fig. 4C) more of this mark had become enclosed in the foregut. The part which lay farthest forward was mainly adherent to the roof and had moved anteriorly since the first inspection.

Behind this a small clump of carbon was discovered on the floor at one side. Farther posteriorly still, carbon was found centrally in the foregut floor. At the level of the anterior intestinal portal particles were again found on the lateral border of the developing floor. In this region the mark had moved posteriorly, for it lay behind the external reference marks. The displacement of the posterior carbon line showed again that a backward movement had again occurred along the primitive streak and the adjacent area pellucida. A few marked cells in the midline, however, had apparently moved only a short distance posteriorly, an unusual occurrence.



TEXT-FIG. 5. Diagram showing: A, a line of carbon across the presumptive foregut area; B, an early stage of foregut formation with the enclosed carbon in a V-formation; and C, a later stage with the enclosed carbon arranged in a diamond shape.

These four specimens are characteristic of 20 marked with transverse lines, 9 at the L stage and 11 at the *h.p.* stage. In every case a backward movement took place in the posterior part of the area pellucida; usually the maximum displacement was along the hinder half of the primitive streak. In specimens which were marked at the L stage across the anterior end of the primitive streak, regression was greatest in the regions just lateral to the midline (2 specimens) or at the edge of the area pellucida (4 specimens).

Specimen No. 2 demonstrates the relative immobility of the endoderm lying just behind the primitive node at the L stage. A similar result was shown by six other specimens marked in the same region. The forward movement which occurred in the head process region was less well marked than the displacement which took place at the posterior end of the area pellucida; yet in eight out of twelve specimens where marks were placed just anterior to the primitive node in the L stage, or across the head process in the *h.p.* stage, a forward movement was found to occur. Three of these specimens were examined every 4 hours after marking, and it was found that the forward movement not only took place in the flat presumptive foregut but was continued in the developing foregut itself. If development had not proceeded far the enclosed carbon was usually arranged in a V-formation (Text-figs. 1B, 4B; schematized in Text-fig. 5 A and B), the anterior medial part being in the roof, the more posterior lateral parts in the floor. With further development the mark on the presumptive floor at each side of the anterior intestinal portal was brought into the midline to meet its fellow from the opposite side (Text-fig. 4C). The linear carbon mark inside the foregut had at this stage become disrupted. Had it stayed complete it would presumably have been transformed from a V-shape to a diamond-shape (schematized in Text-fig. 5C).

TEXT-FIG. 6. Specimen No. 5. The area pellucida only is shown, since the mark did not extend beyond it. A. Position of carbon mass placed on the endoderm of an *h.p.* stage blastoderm. B. The same mark after 5 hours' incubation. C. The same mark after 8 hours' incubation.

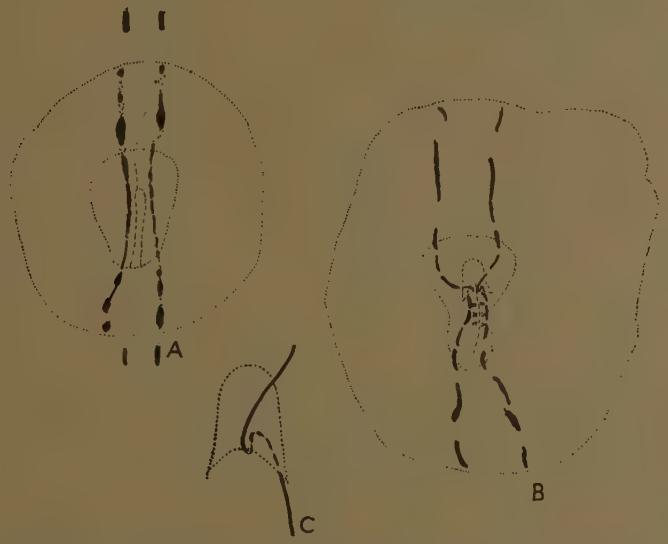
2. *Marks parallel to the primitive streak and the head process*

a. Marks placed on the primitive streak endoderm. These marks were placed on the endoderm in the medial part of the presumptive foregut area (Bellairs, 1953). They thus lay on the presumptive foregut roof.

Specimen No. 5 (*h.p.* stage; *p.s.* 1·8 mm.; *h.p.* 0·5 mm.; *a.p.l.* 2·9 mm.). The head process and the anterior half of the primitive streak were covered with a single, continuous carbon mark (Text-fig. 6A). *After 5 hours* (Text-fig. 6B) the primitive node had regressed farther. The arrangement of the marks indicated that a mediolateral movement had occurred in the endoderm. *After 8 hours* (Text-fig. 6C) the embryonic axis had begun to form and the carbon was

distributed along the roof and the presumptive roof of the developing foregut. Towards the posterior end of the primitive streak a few of the marked cells had migrated farther laterally, whilst others had undergone the usual regression.

Ten specimens in all were marked in this way; they were examined and redrawn to scale every 2–3 hours. In each case a small mediolateral movement was found to have taken place in the endoderm. This was most evident in the mid-primitive streak region of the head process stage. This slight mediolateral movement seldom exceeded 0·3 mm. at either side of the primitive streak.



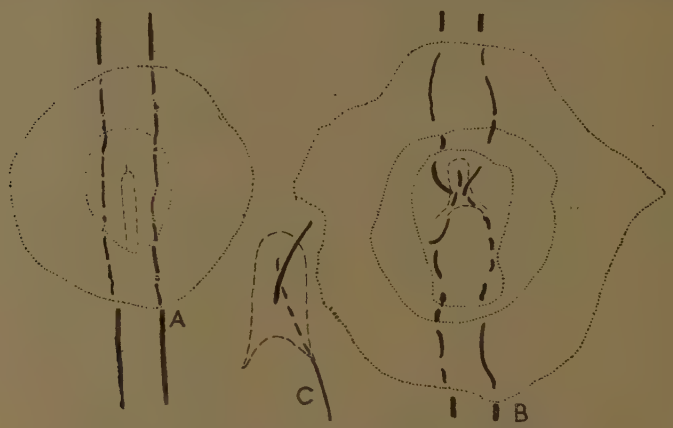
TEXT-FIG. 7. Specimen No. 6. A. Position of two carbon marks placed on the endoderm of an *h.p.* stage blastoderm. B. The same marks after 12 hours. Three pairs of somites have developed. C. Schematic diagram to show the arrangement of the carbon on the left side in the foregut region. The structure outlined is the head in ventral view. Broken lines show the mark in the foregut floor.

b. Marks placed lateral to the primitive streak. These marks passed through the lateral borders of the presumptive foregut area (Bellairs, 1953), i.e. presumably the presumptive foregut floor.

Specimen No. 6 (*h.p.* stage; *p.s.* 1·6 mm.; *h.p.* 0·5 mm.; *a.p.l.* 3·0 mm.). Two lines of carbon were placed parallel to the primitive streak and head process and about 0·2 mm. to 0·3 mm. from it, one on either side (Text-fig. 7A). *After 12 hours* the area opaca had expanded and covered the external reference marks. A lateromedial movement appeared to have taken place in the foregut region, however (Text-fig. 7B). The arrangement of the carbon is shown diagrammatically in Text-

fig. 7C. The anterior part of each mark lay along the yolk-sac endoderm ventral to the head. It passed through the medial part of the anterior intestinal portal into the foregut floor and traces of it were found some distance forward. The marks were traced out of the lateral border of the anterior intestinal portal and continued posteriorly, one on either side of the developing somites.

Specimen No. 7 (*h.p.* stage; *p.s.* 2·2 mm.; *h.p.* 0·7 mm.; *a.p.l.* 3·5 mm.). Two parallel lines of carbon were placed at about 0·4 mm. from the primitive streak



TEXT-FIG. 8. Specimen No. 7. A. Position of two carbon marks placed on the endoderm of an *h.p.* stage blastoderm. B. The same marks after 24 hours. C. Diagram to show the arrangement of the carbon on the left side in the foregut region. Dotted lines show the mark in the foregut floor.

on either side (Text-fig. 8A). *After 24 hours* (Text-fig. 8B) a latero-medial movement had occurred on either side of the anterior part of the developing axis, the anterior part of each mark extending along the extra-embryonic endoderm ventral to the head and almost meeting its fellow in the midline. A small portion of each mark was enclosed in the foregut, but stretched a little farther anteriorly than the posterior end of that which remained on the yolk sac. Most of the enclosed carbon lay in the foregut floor, but some particles were found in the roof as well. In this place, however, a large clump of carbon adherent to the floor lay in the lumen of the foregut. It seemed likely therefore that the roof had become marked secondarily after the foregut had formed.

It is possible that the loss of continuity which occurred between that part of the mark which remained on the yolk sac and that which became included in the floor of the foregut was an artefact brought about by the stretching of the marked region. It seems more probable, however, that the arrangement of the carbon demonstrates an advance in development on that shown by specimen No. 6. Thus it may be that as each side of the foregut floor had become fused, level for

level with the other side, the floor in the midline had lost contact with the extra-embryonic endoderm with which it was previously continuous, the splanchnic mesoderm becoming interposed between floor and yolk-sac endoderm (Text-fig. 8 B and C).

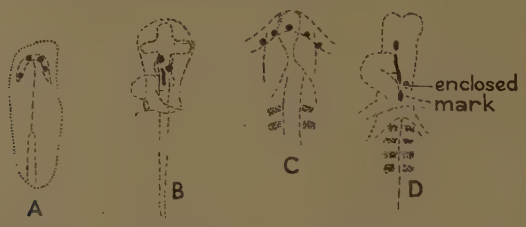
Eighteen specimens were marked in this way and all demonstrated the latero-medial movement in the endoderm. These movements appeared to be closely correlated with the actual ventral closure of the gut itself, since the longitudinal marks were displaced medially as they became enclosed in the floor of the developing organ. Inspection of specimen No. 6 suggests that the carbon which came to lie ventral to the anterior part of the head in the yolk-sac endoderm was derived from the area pellucida anterior to the presumptive foregut area as determined by Bellairs (1953). The more posterior, medially situated yolk-sac endoderm, however, appears to have arisen from a more posterior and lateral position (specimen No. 7). In fifteen of these specimens which were examined every 2 hours during the course of the experiment it was found that no mediolateral displacement took place at the edges of the presumptive foregut area prior to ventral closure of the foregut. On the other hand, in two specimens in which longitudinal marks were placed well outside the presumptive area a mediolateral movement was found to occur; each mark came to lie along the area pellucida border and remained there. In eight cases the posterior ends of the marks on the area opaca moved laterally, and in four of these a similar movement took place in the anterior end. In two specimens only was there a convergence toward the midline of the endoderm at the posterior end of the primitive streak. It is possible that these mediolateral movements in the area pellucida were merely an expression of the lateral expansion of the blastoderm as a whole.

The relationship of the transverse movements just described and the longitudinal migrations shown by the experiments using transverse marks will be discussed later. To investigate more fully the mechanism of closure of the foregut, however, a further series of marking experiments are described below. In the head-fold stage a V-shaped ridge of endoderm forms the ventral border of the anterior intestinal portal and projects backwards on either side of it. It was not clear whether the latero-medial movement simply directed the two arms of the ridge as such into the midline where they joined to form foregut floor and yolk-sac roof, or if in addition there was a rolling in of cells over the ridge; in other words, it was not known whether the ridge represents the limit of the presumptive foregut or not. This problem was tackled by additional marking.

3. *Marks placed on the ridge*

Specimen No. 8 (Head-fold stage; *p.s.* 1·9 mm.; *h.p.* 1·0 mm.; *a.p.l.* 3·6 mm.). Four small carbon marks were placed on the endodermal ridge bordering the anterior intestinal portal (Text-fig. 9A). They were inspected and drawn at intervals of 2 hours. *After 24 hours* a well-proportioned embryo had formed, and the

carbon lay on the yolk-sac endoderm. None of the marks had become enclosed (Text-fig. 9B). The two which were placed lateral to the anterior intestinal portal were subsequently found in a medial position and were somewhat elongated. The two marks which originally lay on the ventral (anterior) border of the ridge had, however, moved only slightly farther medially.



TEXT-FIG. 9. A. Specimen No. 8. Position of four carbon marks placed on the endoderm around the anterior intestinal portal. B. The same marks after 24 hours. The marks all lie on the yolk-sac endoderm. C. Specimen No. 10. Position of five carbon marks placed on the endoderm around the anterior intestinal portal. D. The same marks after 24 hours. Only one mark has become enclosed in the foregut (diffuse shading).

Specimen No. 9 (Early embryo with 2 pairs of somites; *p.s.* 2·2 mm.; *h.p.* 1·7 mm.; *a.p.l.* 4·0 mm.). Five marks were placed on the endodermal ridge around the anterior intestinal portal (Text-fig. 9C). They were inspected and drawn every 2 hours. *After 24 hours* only one mark had become included, and that had originally been placed on the extreme edge of the ridge (Text-fig. 9D).

Nine specimens were marked in this way, and in only two cases (specimen No. 9) did there appear to be a rolling in of a mark over the ridge. Marks placed on the ventral border of the ridge moved only slightly medially. Marks placed on the lateral parts of the ridge, however, were directed bodily into the midline, where they were subsequently found in the yolk-sac endoderm. Some elongation of such marks in an antero-posterior direction was frequent. It seems therefore that, on either side, the lateral limits of the presumptive foregut area coincide with the ridge.

DISCUSSION

Waddington (1952) has drawn attention to the fact that distortion of morphogenetic movements may occur when experiments are made like the present ones in tissue culture, even though an apparently normal embryo develops. For this reason it is unfortunate that direct marking of the endoderm *in ovo* is at present technically impossible. A second disadvantage of the method is that carbon particles, though excellent when used for marking a small group of cells (Bellairs, 1953), are not completely satisfactory when a large tract of the blastoderm

is to be covered. In addition to the discontinuities which develop between individual clumps of carbon, it is difficult with such extensive marks to prevent some of the many granules present from becoming dislodged from the endoderm (e.g. specimen No. 7). Whilst these are frequently washed away at fixation, their presence may lead to error in interpretation. Despite these difficulties, the present results have been obtained with considerable regularity and receive support from earlier observations (Pasteels, 1937; Spratt, 1937; Bellairs, 1953).

The movements which occur in the endoderm in the earliest stages in the formation of the foregut may be classified broadly as:

1. '*Two-dimensional movements*', that is the shiftings of tissues which take place more or less in the original plane of the endoderm (Text-fig. 9, unbroken arrows). These movements consist of migrations of groups of cells in the presumptive foregut roof and in the hinder part of the area pellucida. In addition, the blastoderm as a whole expands radially.
2. '*Three-dimensional movements*' (Text-fig. 9, broken arrows), that is the movements which ultimately lead to folding and ventral closure of the foregut.

In the experiments described above the following 'two-dimensional' movements have been identified. Firstly, there is a forward movement of the endoderm in the developing head process region; this is illustrated by specimens No. 1 and No. 3. A small region toward the anterior end of the primitive streak in the L stage, however, appears to move neither anteriorly nor posteriorly, as in specimen No. 2. Specimen No. 1 illustrates that the endoderm beneath the anterior border of the primitive node is not included in this area but migrates forward.

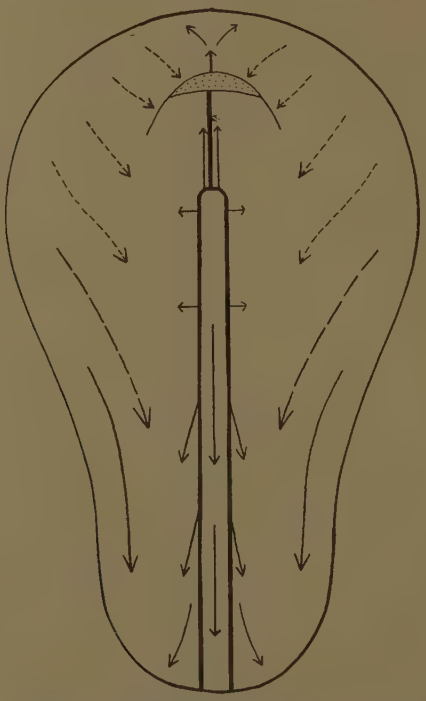
Secondly, there is an extensive regression in the endoderm of the posterior half of the area pellucida, some of the midline cells moving as much as 1 mm. from the middle of the primitive streak to its posterior end. The regions at either side of the midline undergo a similar movement, though usually not so extensively (20 specimens, e.g. Nos. 1, 3, and 4).

Thirdly, some evidence has been presented for a slight mediolateral movement in the endoderm on either side of the primitive streak (e.g. specimen No. 5). It is possible that one or more of these movements may be responsible for, or contributory to, the thinning of the presumptive foregut roof which takes place about this time (Bellairs, 1953).

Fourthly, there is a continual radial expansion of the blastoderm as a whole as it spreads over the yolk. This is incorporated in, and affects, all the other tissue movements occurring in the blastoderm. In the endodermal layer it is demonstrated especially by certain mediolateral movements described in section 2*b* of the results.

Lateral to the presumptive roof region there is an oblique and backwardly directed 'three-dimensional' movement at each side in the area pellucida endoderm (Text-fig. 10). This has been deduced from a study of the transverse and

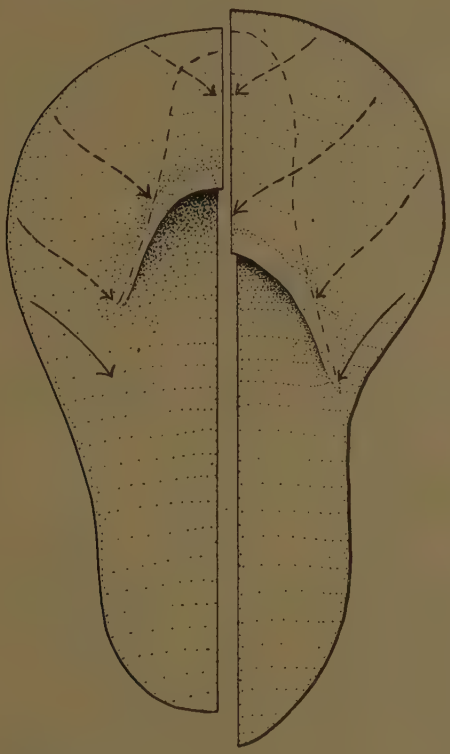
longitudinal shifts of carbon marks which have been placed longitudinally and transversely respectively. Lying originally in the flattened endoderm, the tissues which are destined to form the floor of the foregut are gradually swung medioventrally by the oblique movements. Thus the latter, which at first are of a 'two-dimensional' character, gradually become 'three-dimensional.'



TEXT-FIG. 10. Diagram showing the morphogenetic movements occurring in the area pellucida endoderm during foregut formation. *Broken arrows* show 'three-dimensional' movements. *Unbroken arrows* show 'two-dimensional' movements. The stippled region shows developing foregut. Thick black lines show the position of head process and primitive streak.

The first steps in the formation of the foregut in the early head-fold stage embryo are probably due to the forward migration in the midline combined with the backwardly directed oblique movement of the material on either side. It is probable that the forward movement results in the accumulation of more endoderm at the anterior end of the head process than can be accommodated in a single flattened sheet. Such a situation could lead to the development of a fold of the tissue layer; this would be crescentic, arching forward medially and taper-

ing out laterally (Text-fig. 10). Similarly, the material brought in from the sides by the oblique movements would probably result in the formation of folds at right angles to their direction. The two oblique folds and the transverse fold



TEXT-FIG. 11. Diagram of two stages in the ventral closure of the foregut showing the importance of the 'three-dimensional' movements. The endoderm is seen from its ventral side. Broken arrows represent 'three-dimensional' movements. The anteriormost one on the left side and the two most anterior on the right show oblique movements which have already taken place. Unbroken arrows show 'two-dimensional' movements. The curved broken lines indicate the position of the foregut which is concealed by the yolk-sac endoderm.

would thus enclose a small diverticulum, the anterior end of the foregut. Because of the close fusion between the medially situated endoderm and the overlying mesoderm (Adelmann, 1922) the forward moving tissue would tend to stay dorsal to the material brought in from the sides; the latter lies ventral to loose

mesenchyme. The posterior border of the endodermal pocket formed in this manner would be horseshoe-shaped with backwardly projecting arms, that is, it would have the characteristic outline of the ridge which flanks the anterior intestinal portal.

As development proceeds, the oblique movements take place at progressively posterior levels and in this way the ventral closure of the foregut gradually spreads backwards (Text-fig. 11). Marks placed on the ventral lip of the anterior intestinal portal (see section 3 of the results) show that there is no rolling in of cells medially over the ridges which extend back from the anterior intestinal portal; that is, the oblique movements direct the ridges bodily into the midline. Here they fuse together to form the keel of the foregut floor and this becomes separated from the subjacent extra-embryonic area pellucida endoderm, the two sides of the latter also becoming continuous. The three-dimensional oblique movement merges into the two-dimensional longitudinal movement in the posterior end of the area pellucida. This backward movement is part of the mass migrations of endodermal cells which take place in the hinder part of the blastoderm, marks placed in the middle of the area pellucida being subsequently discovered at its posterior borders (these are among the 'two-dimensional' movements described above). The absence of the oblique movements posteriorly explains why in an embryo with several pairs of somites the foregut closes ventrally only in the anterior half of the area pellucida.

The oblique movements believed to be responsible for the ventral closure of the foregut do not appear to have been described before. The displacements occurring in the endoderm at the hinder end of the area pellucida, however, have not escaped notice. Pasteels (1937) carried out vital marking experiments using vital dyes *in ovo*. Where the mark remained a discreet unit, a movement involving all the layers was deduced; that is, these displacements were occurring in the endoderm as well as in the superficial layers. Although Pasteels's schemes deal primarily with blastoderms at the primitive streak and earlier stages, he includes one diagram representing the *h.p.* stage. The combined movements shown in this map consist essentially of a backward extension of the posterior end of the area pellucida. This receives full support from the present work. In Pasteels's map there is a convergence towards the midline at the extreme posterior end of the area pellucida. This phenomenon occurred also in my specimens as in two cases referred to in section 2*b* of the results. Usually, however, the marks placed in this region were found to have moved laterally.

The extensive backward movement which I have suggested takes place along the endoderm beneath the primitive streak is represented by Pasteels as a phenomenon of the superficial layer only, although his fig. 17 (specimen 14) is strongly suggestive of a regression having occurred in the endoderm. Against this may be placed the evidence of Bellairs (1953), who reported that in certain specimens endoderm cells marked with carbon particles as they lay in the posterior half of the primitive streak region (actually in area F. 3 in the terminology used) were

sometimes subsequently found at the posterior end of the area pellucida, and the similar findings of Spratt (1947) who marked blastoderms with Nile blue sulphate and neutral red.

It is possible that in the normal embryo some of the mass movements of the mesoderm and endoderm are similar at these stages and take place simultaneously. Unfortunately, however, despite the valuable investigations of Gräper (1929), Wetzel (1929), Pasteels (1937), and Spratt & Condon (1947), there is still some doubt as to the exact course taken by the migrating mesoderm during the early stages of organ formation. Pasteels (1937) and Spratt (1947) have given evidence of the backward migration of the primitive node and of the regions lateral to the primitive streak, and Spratt believes that this is contemporaneous with that of the endoderm. He carried out a number of experiments using vital dyes in a manner similar to that of Pasteels (1937) and reported that at least along the primitive streak itself there seemed to be 'no differential displacement of one layer relative to another'.

During the backward movement the endoderm is so closely applied to the mesoderm that a simultaneous movement of the two layers might be expected. There is not, however, a complete correspondence of movement between the mesoderm and endoderm during regression, for whereas the primitive node is involved in the backwards migration of the mesoderm (Wetzel, 1929; Pasteels, 1937; Spratt, 1947), the present experiments suggest that the endoderm of that level remains in relatively the same place.

Certain deductions as to the movement of the anterior part of the splanchnic mesoderm may also be made as a result of the present work. It seems likely from the study of serial sections that the close association which exists between the endoderm and the splanchnic mesoderm is retained throughout this period. Except in the region of the cardiac vesicles the contours of the thickened gut wall are closely followed by those of the similarly thickened splanchnic mesoderm at all stages in the formation of the closed gut. It is not improbable, therefore, that the two layers undergo simultaneous and identical movements; that is, the oblique lateral movement is common to both.

The forces which actually initiate and control the tissue movements in the chick blastoderm at this stage are but little understood. The close association of the developing head fold and the anterior end of the foregut is well known and has led many authors to imply that the presence of one is responsible for that of the other, e.g. Lillie (1952) states: 'The head fold thus produces an internal bay in the endoderm, the beginning of the Foregut.' The independence of the development of the foregut and the formation of the head fold has, however, been demonstrated (Waddington & Cohen, 1936; Abercrombie & Waddington, 1937) and I have obtained specimens which confirm it. A further point is that, even were the head fold responsible for mechanically initiating foregut formation, some other influence must also be sought, since the ventral closure of the foregut rapidly outstrips the backward extension of the head fold.

SUMMARY

1. The morphogenetic movements which take place in the endoderm of the chick during foregut formation have been traced by means of carbon marking on blastoderms explanted *in vitro*.

2. Two types of movement are distinguished, called 'two-dimensional' and 'three-dimensional', the former occurring in the original plane of the endoderm, the latter folding certain regions medio-ventrally to form the floor of the foregut.

3. The most anterior tip of the foregut is believed to be formed as a pocket between two sets of opposing movements in the endoderm: (*a*) a forward, two-dimensional movement beneath the head process, and (*b*) an obliquely backward, three-dimensional movement on either side.

4. During the formation of the foregut the oblique movements result in a U-shaped ridge in the endoderm bordering the anterior intestinal portal. These movements spread progressively backwards and result in the two limbs of the ridge being brought together in the midline, where they fuse and thus gradually close off the cavity of the foregut ventrally from that of the yolk sac.

5. Some forward movement takes place at the anterior end of the developing foregut.

6. In the posterior half of the area pellucida there are extensive displacements of endoderm cells in a posterior direction.

ACKNOWLEDGEMENTS

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On the Significance of the Neuromeres and Similar Structures in Vertebrate Embryos

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Head: Professor Gösta Glimstedt.

WITH THREE PLATES

INTRODUCTION

ABOUT the time of closure of the neural folds, transverse bulges—so-called proneuromeres (Källén & Lindskog, 1953)—appear in the wall of the central nervous system of vertebrate embryos. They are very similar to the well-known neuromeres, but are relatively larger and therefore fewer in number. The first proneuromeres to develop are the rostralmost ones and the others develop in a rostro-caudal sequence. They start to disappear simultaneously at two places: the caudal end of the spinal cord and a short-distance caudal to the otic vesicle. When the proneuromeres have disappeared in the spinal cord and the caudal part of the brain but are still left most rostrally, the neuromeric bulges develop. The neuromeres have been described many times in the previous literature (see Bergquist's survey, 1952*b*). They develop and disappear in a similar way to the proneuromeres (Bergquist, 1952*a*; Källén & Lindskog, 1953). While the neuromeres are disappearing a third system develops: the migration areas. They form what we called 'transversal bands', within which there are sometimes rather shallow ventricular furrows, and between which external fissures have been described in the early stages of the migration areas (Bergquist, 1952*c*; Bergquist & Källén, 1953*c*). One or two of the neuromeres correspond to each proneuromere, and one, two, or more 'transversal bands' correspond to each neuromere (Källén & Lindskog, 1953; Bergquist & Källén, 1953 *a* and *b*).

From studies by, amongst others, Coghill (1924) and Hamburger (1948) it is known that there are often centres of mitotic activity present in the central nervous system. Coghill described a transverse patterning in the brain and Hamburger a significant difference in mitotic activity between the dorsal and ventral halves of the spinal cord. The present author (1952) has shown that the neuromeric ventricular furrows (*sinuationes*) lie in the middle of proliferation centres. Bergquist (1932), Rudebeck (1945), and Källén (1950) have shown that

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this is also true for the ventricular furrows of the migration areas. It seems likely also that the proneuromeres coincide in position with proliferation centres. These facts have directed the author's interest to the relation between the proliferation processes and the bulges of the brain wall. There seem to be four different ways of explaining the coincidence in position between the proliferation centres and the bulges: (1) The proliferation processes and the bulges are independent but are both caused by the same 'segmentation' factor. (2) The bulges are formed first and stimulate in some way the proliferation processes, so that there are peaks of proliferation in the middle of them. (3) The proliferation occurs in such a way as to form groups of cells, which build the bulges. The bulges disappear when new cells are formed in the regions between the bulges, e.g. around the interneuromeric fissures. (4) The bulges are only signs of temporary local increases in proliferative activity, and the disappearance of the bulges takes place when the proliferation has faded.

The author has tried to find out which explanation is the most likely one by influencing proliferative activity with colchicine. The experiments were first tried on neuromeric stages. The results, however, were very difficult to analyse. The neuromeres are formed and disappear very rapidly and it is therefore difficult to decide whether an experimentally obtained deep neuromere merely represents a maximally developed normal one. It is also well known that young mammalian embryos of the same age—belonging to the same litter—may be rather differently developed. For these reasons it is very difficult to match the experimental embryos with normal stages of the same degree of development.

The experiments were therefore carried out chiefly on older embryos, at the stages with migration areas, where the degree of development of embryos of a certain age is relatively uniform. The results of these experiments are also easier to analyse, as the proliferation furrows in normal development never become very deep.

MATERIAL AND METHODS

The study was carried out on *Mus musculus*. 0·025 mg. of colchicine was injected subcutaneously into each gravid female. Three hours later the animals were killed, the embryos removed and fixed in Bouin's fluid. The embryos were sectioned transversely and stained with haematoxylin and eosin. Some of the embryos and some normal embryos of the same stage were reconstructed by the wax-plate method. The age of the embryos was determined in the following way. The male and female mice were put together for 1 hour when the female was in pro-oestrous phase. If sperms could be detected in the vaginal smear afterwards, the female was considered to be fertilized. The experimental embryos used are listed in Table 1. For comparison the normal mouse material of the Tornblad Institute has been used—many series of the same age as the experimental embryos and also younger and older stages.

TABLE 1

Mus musculus *var.* alb. *Experimental embryos*

Number in Tornblad collections	*Age when killed*	*Reconstructions*
82	9 d. 6½ h.±½ h.	
83	9 d. 6½ h.±½ h.	
86	9 d. 8 h.±½ h.	
71	9 d. 8 h.±½ h.	
84	11 d. 8½ h.±½ h.	Wax-plate 50×
91	11 d. 8 h.±½ h.	
92	11 d. 8 h.±½ h.	
95	11 d. 8 h.±½ h.	Wax-plate 50×
98	11 d. 8 h.±½ h.	Wax-plate 50×

RESULTS

As is well known from numerous studies on the influence of colchicine on mitotic activity, this substance arrests mitoses in early metaphase and thus prevents their development into telophase. That a colchicine effect was produced in the mouse embryos with the concentration used is easily seen by the morphology of the mitoses. In the normal embryos mitoses are seen in anaphase and telophase, in the experimental ones there are none in stages later than metaphase. Paff (1939) described an over-production of cells as a result of colchicine treatment of chick embryos. The present author has not obtained this effect in his experiments, perhaps because of differences in the dosage used.

If the mitoses are arrested irreversibly in their development it seems to follow that there will be an accumulation of mitoses in the proliferation centres, because during the 3 hours of the colchicine treatment new mitoses must start to develop and become arrested. How do these changes in proliferative activity influence brain morphology? A comparison between sections and models of experimental and normal brains shows that the former are all changed in a similar way, though the changes may be of different degree in different embryos. The changes are of a nature never found in normal development, and are illustrated by the following specimens.

If the models of the brains of experimental embryos (series 95 and 98, Plate 1, figs. A and B) and of a normal embryo (series 100, Plate 1, fig. C) are compared it is apparent that the main configuration is the same, but the parietal flexure is more developed in the experimental animals. This seems to be an effect of a greater development of the mammillary recess, a bulging of this part of the brain wall towards the rhombencephalon. All other ventricular bulges show a similar phenomenon. The diencephalic sulci, which can hardly be seen on the model of the normal brain (Plate 2, fig. C), though they are visible in the sections (Plate 3, fig. B), are very deep in the experimental embryos and cause external bulges of

the wall (Plate 2, figs. A and B). The difference as it appears in sections through the diencephalon is shown in Plate 3, figs. A and B.

The diencephalic furrows in the experimental animals are distinctly reminiscent of neuromeric furrows (*sinuationes*). The lateral surfaces of the brains also suggest a series of neuromeres, since the normally very indistinct external limiting fissures between the 'transversal bands' are greatly deepened and simulate interneuromeric fissures. The mesencephalic bulge is also strongly deepened in the experimental embryos.

In the rhombencephalon there are no ventricular furrows in the normal embryos (Plate 1, fig. C; Plate 3, fig. C). In the experimental embryos furrows are present (Plate 1, figs. A and B; Plate 3, fig. D). They are, however, not deep enough to cause external fissures in this brain region.

DISCUSSION

From these observations the following conclusion can be drawn: colchicine has produced a strong deepening of the ventricular furrows so that these simulate neuromeres, and has caused the development of furrows in a region where normally none are visible (in the rhombencephalon). This emphasis of the furrows has taken place without the formation of new cells—all mitoses are arrested by the colchicine.

Besides the well-known effect of colchicine on mitoses—their arrest in metaphase—it might be argued that some more unspecific effect may have caused the changes of the morphology of the brain wall. Changes in the stainability of the neuroblasts, the structure of the connective tissue, &c., are indeed seen. These unspecific effects, however, are present all over the embryos, but the morphological changes of the brain wall are restricted to the proliferation centres. As no specific colchicine effect on the proliferation centres is known other than that referred to above, it seems most likely that the latter is the cause of the bulging of the wall.

If, as seems very probable, changes in the process of proliferation influence the bulges of the wall, the first and second explanations proposed on p. 388 must be incorrect. As no new cells were formed in these experiments, the third explanation also cannot be right. The results, however, fit well the fourth and last explanation. The bulges of the nervous system are signs of increased numbers of mitotic cells and they disappear when the numbers of mitotic cells decrease again.

How, then, shall this phenomenon be explained? As Sauer (1935) has described, the 'germinal cells' of the embryonic nervous system are neural epithelial cells that have migrated to the ventricular surface and have there gone into mitosis. After the mitotic process the daughter cells again migrate laterally (cf. Hamburger & Levi-Montalcini, 1950). The mitoses, arrested by colchicine in the experiments above, remain at the ventricular surface and meanwhile new cells migrate to that surface and go into mitosis. In this way the

proliferation centres will contain more mitoses than normally. The ventricular surface will then also be too large and will bulge. Why the bulge is lateralwards and not medialwards seems to be a question of pressure conditions.

The appearance of the furrows in normal development may now be explained. When a proliferation centre is formed—i.e. when the proliferation is intensified somewhere—the same process must take place and the wall must bulge. As soon as the proliferation intensity has faded, the bulge will cease to exist. In this way the ventricular furrows and bulges represent signs of local intensifications of proliferation.

The three systems of transverse bulges previously described now acquire another significance. They are signs of three successive processes of proliferation acceleration. At least the first two of them (causing proneuromeres and neuromeres) develop in rostro-caudal sequence. This result fits well with the conclusion reached by Coghill (1924) that a rostro-caudal wave of proliferative activity occurs in *Ambystoma*.

SUMMARY

1. The relation between certain transverse bulges of the wall of the central nervous system (proneuromeres, neuromeres, and 'transversal bands' of migration areas) and proliferative processes was studied. Embryos with early migration areas were treated with colchicine and in this way the proliferative activity was changed.

2. The number of cells in mitosis is increased by the colchicine and as a result of this the depth of the bulges is increased. The normally very shallow ventricular furrows come to resemble neuromeric furrows.

3. The occurrence of the bulges in normal development is therefore probably the result of local increases of proliferative activity, i.e. of the formation of proliferation centres. It is suggested that all three systems of transverse bulges (proneuromeres, neuromeres, and 'transversal bands') represent successive activations of transverse proliferation centres.

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EXPLANATION OF PLATES

Abbreviations: *fiss. int-mes.*, intermesencephalic fissure (between transversal bands 6 and 7); *ggl. V*, ganglion trigemini; *Hem.*, hemispherical evagination; *isthm.*, isthmic fold; *opt. stalk*, optic stalk; *s. 6–11*, ventricular sulci within transversal bands 6 to 11; *s. c. th.*, sulcus caudalis thalami; *s. com. post.*, sulcus commissurae posterioris; *s. lim. His*, sulcus limitans of His; *s. m. th.*, sulcus medialis thalami; *s. r. th.*, sulcus rostralis thalami; *tr. bnd. 3–7*, tranversal bands numbers 3 to 7; *x*, (in Plate 3, fig. D) newly formed ventricular furrow in the rhombencephalon.

PLATE 1

FIGS. A, B, and C. Reconstructions of the brains in medial view from embryos of *Mus musculus*. The section surface of the brain half is marked with black. Magnification 20×. A. Experimental animal, 11 days 8 hours old, series 95. B. Experimental animal, 11 days 8 hours old, series 98. C. Normal animal, 11 days 8 hours old, series 101.

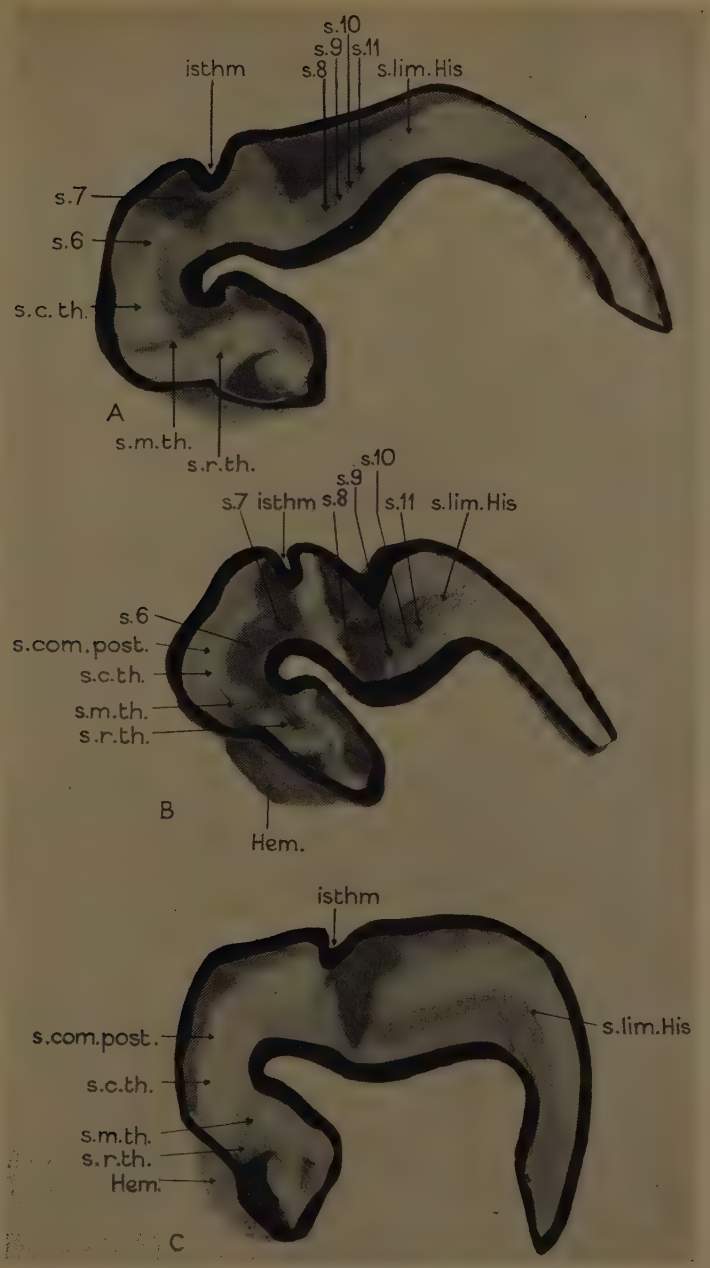
PLATE 2

FIGS. A, B, and C. Reconstructions of the brain in lateral view from embryos of *Mus musculus*. Magnification 20×. A. Experimental animal, 11 days 8 hours old, series 95. B. Experimental animal, 11 days 8 hours old, series 98. C. Normal animal, 11 days 8 hours old, series 101.

PLATE 3

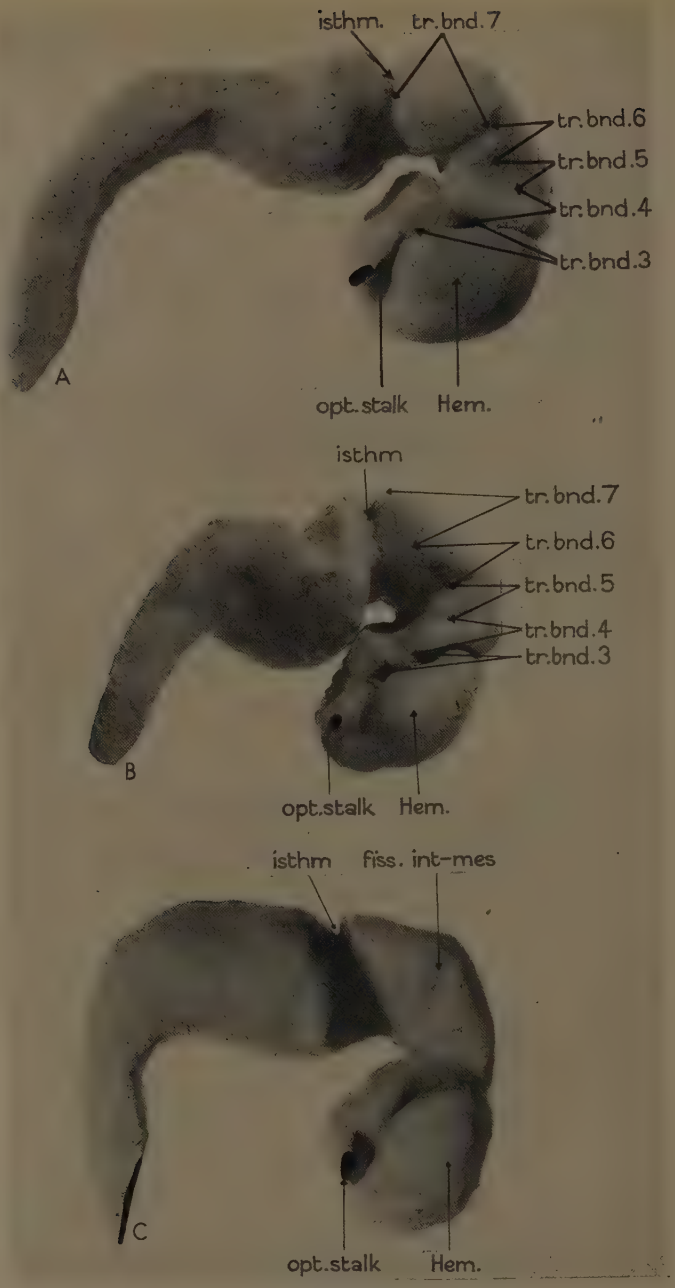
FIGS. A and B. Transverse sections through the diencephalon of *Mus musculus*, stained with haematoxylin-eosin. Magnification 80×. A. Experimental animal, 11 days 8 hours old, series 98. B. Normal animal, 11 days 8 hours old, series 101.

FIGS. C and D. Transverse sections through the rhombencephalon of *Mus musculus*, stained with haematoxylin. Magnification 70×. C. Normal animal, 11 days 8 hours old, series 101. D. Experimental animal, 11 days 8 hours old, series 95.



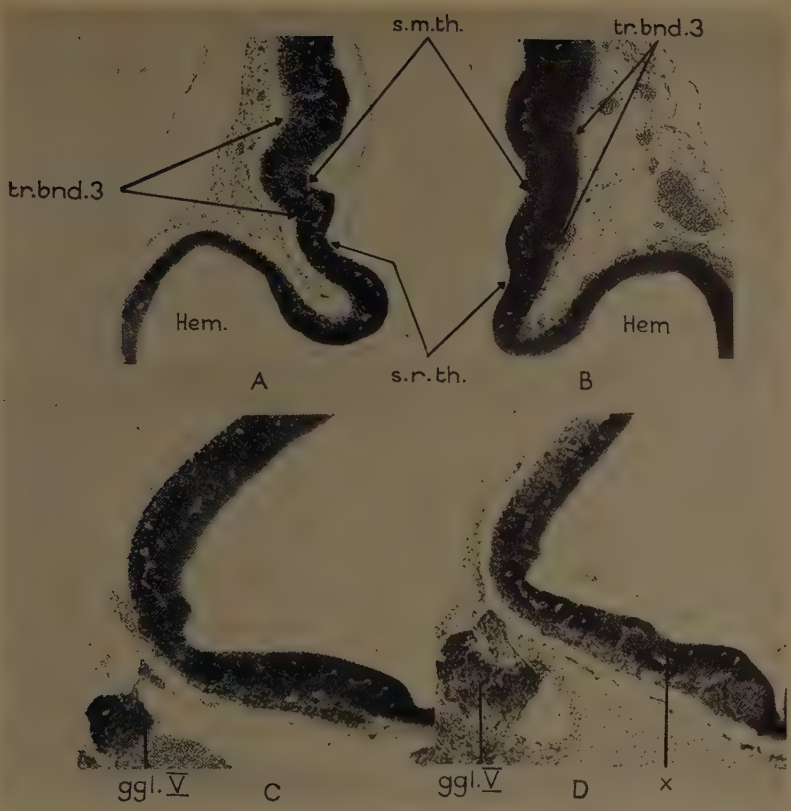
B. KÄLLÉN

Plate 1



B. KÄLLÉN

Plate 2



B. KÄLLÉN

Plate 3

Notes on the Development of the Neural Crest in the Head of *Mus musculus*

by BENGT KÄLLÉN[1]

From the Tornblad Institute for Comparative Embryology, Lund.
Head: Professor Gösta Glimstedt

INTRODUCTION

THE relations between the neural crest on the one hand and the neuromeres and their precursors the proneuromeres on the other hand have never been fully described. Many authors have, however, shown that the cranial ganglia lie level with every second neuromere, so that ganglion V lies level with neuromere VII, ganglion VII–VIII level with neuromere IX, and ganglion IX–X level with neuromere XI (Bergquist, 1952). The neuromeres develop in a rostro-caudal wave, which is preceded by a similar wave of proneuromeres—formations of approximately the same appearance but relatively larger (Källén & Lindskog, 1953). The first proneuromere directly corresponds to neuromeres I–II, the second to neuromeres III–IV, the third to neuromere V; and in the rhombencephalon each proneuromere also corresponds to two neuromeres.

These observations raise the following questions: (1) Does the neural crest differentiate in a rostro-caudal sequence as the proneuromeres and neuromeres do? (2) What are the spatial relations between the proneuromeres and the ganglion anlagen of the neural crest? (3) What is the time relation between the formation of the neural crest and the development of the proneuromeres and neuromeres?

Adelmann (1925) described the development of the neural crest in the rat as a cranio-caudal progression of proliferation. In a 5-somite embryo the neural crest is only developed in the territory of the mesencephalon and the rhombomere A_1 (corresponding to Källén & Lindskog's proneuromere 4), and forms the anlage of the trigeminus ganglion. In the 8-somite stage there is also an anlage of the facialis-acusticus ganglion and of the glossopharyngicus-vagus ganglion, the latter being continuous with the spinal neural crest. Adelmann considered that there is a cephalo-caudal differentiation also in the spinal neural crest, but he thought that this is independent of that of the head neural crest. He observed a more advanced differentiation in the rostral part of the spinal crest than in the glossopharyngicus-vagus crest.

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Adelmann described no neural crest rostral to the mesencephalon. Others, however, have shown that there is probably also crest rostral to the mesencephalon in mammals, though it is very small and difficult to observe with morphological methods (Holmdahl, 1928, and da Costa, 1931). In lower vertebrates such a crest is sometimes well marked. According to Raven (1931) this part in urodeles probably develops later than the mesencephalic crest, and da Costa (1931) found the same in *Cavia*, but according to Wedin (who called this rostral part the thalamic crest, 1949) it is developed earlier than the mesencephalic crest in *Torpedo* (personal communication). These results then show that the neural crest is made up of morphologically separate parts: in the rhombencephalon the ganglion anlagen, and rostral thereto the mesencephalic and thalamic crests. The author will in this paper call these different parts 'neural crest portions'.

Adelmann's results thus seem to answer the first question partly in the affirmative; he thinks that there are two different and independent progressions of cephalo-caudal differentiation in the neural crest, one in the head and one in the spinal neural crest. Conel (1942) found that there was one cephalo-caudal progression extending over both the brain and the spinal cord in selachians.

The present paper provides some additional data on the first question posed above, and attempts to answer the two remaining questions, which have not previously been discussed in the literature on the subject.

MATERIAL AND METHODS

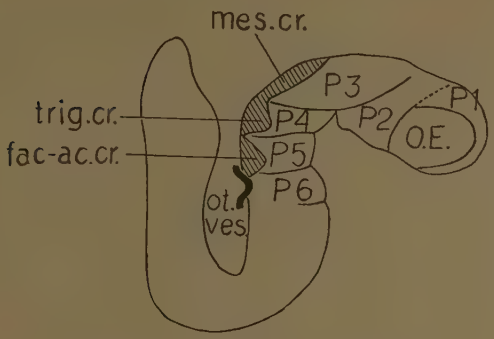
The embryos of *Mus musculus* previously used by Källén & Lindskog (1953) were studied. The wax-plate reconstructions then made were supplemented by graphical reconstructions of the neural crest portions. The staging of the embryos is the same as that of Källén & Lindskog and the reader is referred to that paper for the details of technique.

RESULTS

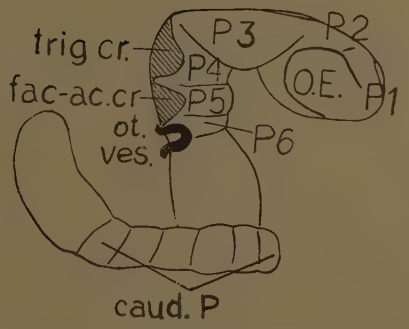
In stage 5 the neural crest is present only in the mesencephalon, and only in its caudal part.

In stage 6 the mesencephalic portion is still present, but caudal to it two other neural crest portions have migrated (Text-fig. 1). The rostralmost one is not clearly separated from the mesencephalic portion and is marked *trig.cr.* in the figure as it represents the anlage of the trigeminal ganglion. The third portion lies just rostral to the otic vesicle and is marked *fac-ac.cr.*, representing the anlage of the facialis and acusticus ganglia. The three portions exactly correspond to proneuromeres 3, 4, and 5 respectively.

In stage 10 (Text-fig. 2) the mesencephalic portion has disappeared—its cells have presumably migrated out into the mesenchyme and cannot be identified. A similar process of dispersion of cells perhaps takes place from the very beginning in the thalamic region, so that no visible crest develops there. The other two portions are still present, and caudal to them lies the otic vesicle.

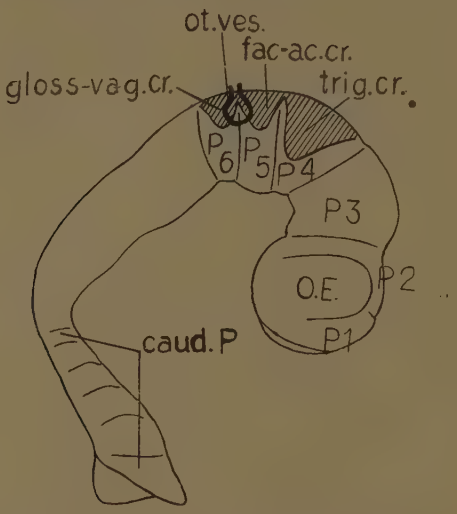


TEXT-FIG. 1. Drawing of a wax-plate reconstruction of the central nervous system of *Mus musculus*, stage 6. In the reconstruction the different portions of the neural crest are marked and hatched. *fac-ac.cr.*, facialis-acusticus ganglion anlage; *mes.cr.*, mesencephalic crest; *O.E.*, optic evagination; *ot.ves.*, otic vesicle; *P 1–6*, proneuromeres 1–6; *trig.cr.*, trigeminus ganglion anlage. Magnification ×65.



TEXT-FIG. 2. Drawing of a wax-plate reconstruction of the central nervous system of *Mus musculus*, stage 10. In the reconstruction the different portions of the neural crest are marked and hatched. Abbreviations as in Text-fig. 1; and *caud.P.*, caudal proneuromeres, impossible to number. Magnification ×50.

In stage 11 (Text-fig. 3) a fourth neural crest portion has migrated, level with proneuromere 6, i.e. caudal to the otic vesicle. No spinal crest is yet formed. The new part of the neural crest is marked *gloss-vag.cr.* in the figure, and is the anlage of the glossopharyngicus and vagus ganglia.



TEXT-FIG. 3. Drawing of a wax-plate reconstruction of the central nervous system of *Mus musculus*, stage 11. In the reconstruction the different portions of the neural crest are marked and hatched. Abbreviations as in Text-fig. 1; and *caud.P.*, caudal proneuromeres, impossible to number; *gloss-vag.cr.*, glossopharyngicus-vagus ganglion anlage. Magnification ×60.

DISCUSSION

These results seem to make it possible to answer the three questions, posed in the introduction, in the following way:

(1) The neural crest differentiates as a cranio-caudal progression as Adelmann believed, but—contrary to his opinion—it seems likely that there is only one cranio-caudal process, extending over the brain *and* the spinal cord, as the spinal crest is not yet developed in stage 11, where the glossopharyngicus-vagus crest is already formed. The author's results thus agree with Conel's (1942) findings on selachians.

(2) The proneuromeres exactly correspond to the neural crest portions of the head. This fact explains why the ganglia later lie level with every second neuromere, since every proneuromere in the rhombencephalon gives rise to two neuromeres.

(3) The neural crest portions develop immediately after the appearance of the corresponding proneuromeres.

These facts make it likely, in my opinion, that there is some causal relationship between the formation of the proneuromeres and the neural crest portions. Results obtained in a previous investigation (Källén, 1953) suggest an explanation of that relationship. It was then shown that the serially arranged transverse bulges of the vertebrate central nervous system are manifestations of serially arranged centres of proliferation. The proneuromeres form the first set of these bulges to develop. At this stage the neural crest is not yet separated from the neural tube. It seems then likely that the factor causing the proliferation centres within the neural tube, which factor is the basis of the proneuromeres, can also operate within the neural crest, producing there proliferation centres level with the proneuromeric ones. From these centres cells may migrate as the different neural crest portions. In later stages the neural crest and the neural tube are not so closely connected, which perhaps explains why the factor forming the subsequent neuromeric proliferation centres does not effect the development of the neural crest.

This explanation seems to fit with the observation, made above, that the proneuromeres develop a little earlier than the neural crest portions. The former are signs of the proliferation centres themselves, the latter probably develop from cells formed by the proliferation processes. It also fits well with the observations made by Schulte & Tilney (1915) on cat embryos and by Conel (1942) on selachians and *Torpedo* that there are small furrows or evaginations in the earliest neural crest anlagen. These might then correspond to the proliferation furrows and the neuromeric furrows of the medullary tube.

Why, then, does not the division of the neural crest continue into the spinal cord as the proneuromeres do? Here a parallel with the formation of the third transverse proliferation pattern in the neural tube (that of the 'transversal bands' of Bergquist & Källén, 1953) can be drawn. These formations are likewise indistinct in the caudal part of the rhombencephalon and disappear in the spinal cord. Some sort of cranio-caudally decremental factor thus seems to exist. The nature of this, however, is intimately associated with the nature of the processes underlying the formation of the proliferation patterns, and a final answer to this question cannot be given until the latter is more thoroughly understood.

SUMMARY

The development of the neural crest in the mouse has been studied in relation to the formation of proneuromeres. The ganglion anlagen develop in a rostrocaudal direction, probably extending over both the brain and the spinal cord. Every anlagen corresponds to one proneuromere and develops a little after the appearance of the corresponding proneuromere.

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The Morphogenesis of the Notochord in Amphibia

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INTRODUCTION

In spite of the great progress of experimental embryology in recent years, we still know rather little about the physical processes by which the various parts of the egg become moulded into definitely shaped organs. It is obvious, however, that this phenomenon of morphogenesis is one of the most important and characteristic aspects of development, and further knowledge about it is much to be desired. The investigations to be reported here represent an attempt to obtain further understanding of such processes as they affect the Amphibian notochord. This organ was chosen because the final shape which is attained is of great simplicity, being no more than an unbranched cylindrical rod. Moreover, casual inspection shows that rather considerable changes in cell shape occur during the formation of this rod, and it appeared possible that these changes in the constituent cells would be found to be directly related to the morphogenesis of the organ as a whole.

MATERIALS AND METHODS

The embryos used were those of Axolotl (*Ambystoma tigrinum*), *Triturus alpestris* (imported from Belgium and Switzerland), and locally caught *Triturus palmatus*.

Material for histological examination was fixed in Bouin's fluid and cleared through xylol or methyl benzoate; small fragments of tissue can be handled quite satisfactorily in xylol provided they are left in the fluid for not more than a few minutes. Most of the material was stained in Delafield's haematoxylin, but some preparations stained in Haidenhain's Azan have also been examined. In a third series the dorsal roof of the embryo was cut off and spread out flat under a strip of coverslip in a Petri dish containing Smith's Formol-Bichromate fixative. The ends of the piece of coverslip were supported by two other fragments of glass so that it slightly compressed but did not crush the tissue. After fixation overnight in this position the tissues remained flat, so that accurate horizontal sections of the whole length of the archenteron roof could be obtained. These

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were much easier to interpret than the usual mixture of transverse, oblique, and horizontal views which results from cutting the curved dorsal layers in the intact embryo. Most of these flattened preparations were stained with Mallory's triple stain by the modified procedure of Cason (1950).

Fragments of notochord tissue were cultivated in a number of different salines. These were all based on 'normal Holtfreter saline', which was made up in the usual way, with the pH adjusted to a value of 7 by means of phosphate buffers (2·3 per cent. KH_2PO_4 with 1·5 per cent. K_2HPO_4). In one series of modified salines the pH was altered by the addition of sodium phosphate buffers as shown

TABLE 1

Solution	Na_2HPO_4	NaH_2PO_4	*pH*
a	9·75+0·1 KOH	0·25	11·2
b	9·75+0·05 KOH	0·25	9·3
c	9·75	0·25	8·2
d	9·0	1·0	7·6
e	7·0	3·0	7·1
f	3·0	7·0	6·4
g	0·28	9·75	5·5
h	0·25	9·25+acetic acid	4·6
i	0·25	9·25+acetic acid	3·2

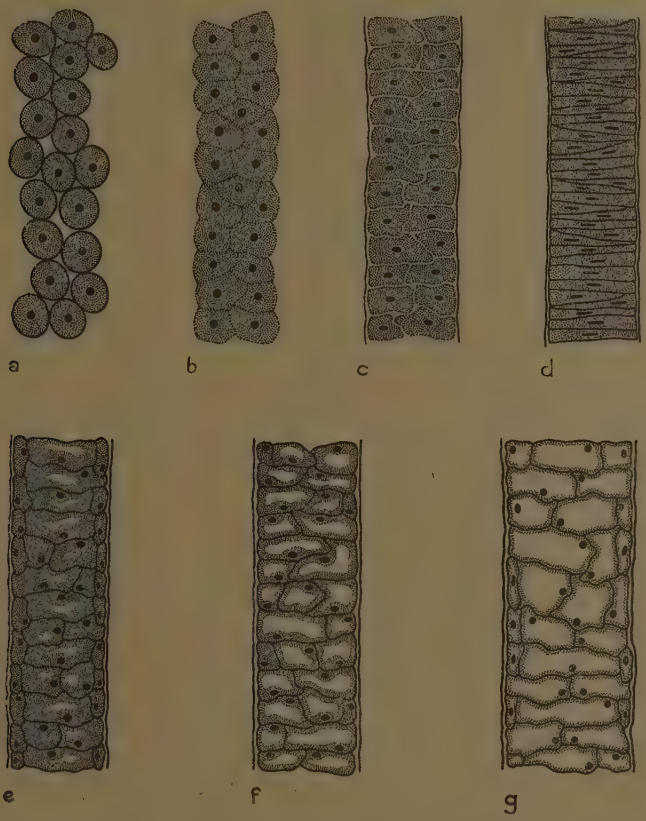
in Table 1; the pH values of these were measured by Dr. H. G. Callan on a Cambridge pH meter. Other salines were prepared by the subtraction or addition of calcium ions in solution *c*, and solutions with altered surface tension were made by the addition of sodium lauryl sulphonate to solution *c*; these are mentioned in more detail in the text.

We should like to express our gratitude to Dr. (now Professor) H. G. Callan for assistance with the pH measurements.

THE DEVELOPMENT OF THE NOTOCHORD *IN SITU*

During the process of invagination the presumptive notochord cells go through the well-known 'flask-shaped' stage which has been described by Waddington (1940, 1942), Holtfreter (1943), and others. After they have passed through the blastopore and moved forward to form the roof of the archenteron, they return to a quasi-spherical shape and adhere to one another rather weakly, so as to constitute a loose tissue (Text-fig. 1*a*). The definitive notochord begins to be formed in the late gastrula by the separation from the archenteron roof of a mid-dorsal strand of tissue. The cells of this arise partly by delamination, that is by active movement out of the general layer of archenteron roof, but to some extent they are formed by mitosis. They are at first more or less spherical, and only loosely attached to one another, forming an elongated strand of tissue two or three cells wide. Soon the cells begin to cohere more strongly, so that it looks as if the strand were being compressed from all sides. The cells alter towards a

polygonal shape in longitudinal section (Text-fig. 1*b*), while the notochordal strand as a whole assumes a roughly circular outline in transverse section.



TEXT-FIG. 1. Diagrammatic drawings of longitudinal sections through the developing notochord. *a*. Loosely adhering, roughly spherical cells in the late gastrula (slit yolk-plug) stage. *b*. More or less polygonal cells, very early neural plate stage. *c*. Closely adhering cells, with hardly visible membranes. First signs of chordal sheath. Mid-neural plate stage in *Triturus* (H. 15), rather later in *Amblystoma* (H.17). *d*. 'Pile of coins' arrangement, early tail-bud stage (H.23). *e*. Beginning expansion of the cells, showing 'chordal epithelium'. Mid tail-bud stage. *f*. Later stage of expansion. *g*. Fully developed larval notochord.

By the early neural plate stage (Harrison's stage 14) the notochordal strand in *Triturus* has already become a definite rod, distinctly separated from the rest of the archenteron roof mesoderm. In this the intercellular membranes are very difficult to see with most stains, but they can be detected in sections as clear

regions from which yolk granules are absent (Text-fig. 1*c*). A similar condition is reached at a slightly later stage (about H. 17) in *Ambystoma*, and in this form the membranes are clearer. In both species the cells are more or less equidimensional polyhedra. At the same time, the presence of the notochordal sheath can first be recognized, as a very thin membrane staining with Aniline Blue.

In the later stages of neurulation rather rapid alterations occur in the shape of the chordal cells. There is in the first place a considerable increase in number; in an early tail-bud stage (H. 23) there are about 50 per cent. more cells per unit length of chorda than in the mid-neurula stage (H. 17). This increase is presumably due to cell division, although mitoses are rarely seen in the sections. The cells are arranged in a highly characteristic way, which may be compared to a pile of coins, many of the cells being reduced to flat disks which occupy the whole thickness of the notochord rod, though some do not extend right across it (Text-fig. 1*d*).

The first stage in the transition to this condition is an increase in the vertical (dorso-ventral) dimensions of the cells, which in sagittal section take an appearance very similar to those of the overlying neural groove. This causes the whole notochord to become oval in cross-section, with the long axis vertical. At the same time the nuclei become extremely flattened anterior-posteriorly. Soon after this the notochord cells increase in width in the medio-lateral direction, so that the cross-section of the notochord becomes circular once more, with most cells extending right across it. The cell membranes become thicker during this process, and stain more deeply, particularly in dyes such as Aniline Blue, and the notochord sheath is also becoming more obvious. The cells are still nearly filled with yolk granules, although these are beginning to disappear in the centre of each cell in the neighbourhood of the nucleus.

The flattened disk-shaped cells of the early tail-bud embryo gradually expand again into a polyhedral shape during the development to the late tail-bud stage. The expansion is brought about by a vacuolization, the cytoplasmic contents of the cells eventually disappearing almost completely except in the immediate neighbourhood of the nucleus. The expansion and vacuolization begins in the centre of the rod, and as the expanding cells move relative to one another, a more or less continuous layer of unexpanded cells, still containing plentiful internal cytoplasm, is formed at the surface of the notochord; this is known as the notochordal epithelium (Text-fig. 1*e*). It is never completely continuous over the whole surface, and as the expansion and vacuolization continue it gradually disappears so that in the fully formed larval notochord all the cells are vacuolar and polyhedral in shape, although the more superficial ones may remain somewhat smaller than those more deeply placed (Text-figs. 1*f*, 1*g*). No mitoses can be seen during the expansion of the cells, and the enlargement of the chorda seems to involve no increase in cell number. It is accompanied by the deposition of the chordal sheath, the formation of which will be discussed in another paper (Mookerjee, 1953).

DIFFERENTIATION OF ISOLATED FRAGMENTS OF NOTOCHORD

(*a*) *In normal saline*

It is well known from the work of Holtfreter (1938, 1944) and others that pieces of presumptive notochord can develop into this tissue after isolation in normal Holtfreter saline. The shape of the chordal masses is, however, always extremely abnormal. When tissue is isolated from young gastrulae, somewhat elongated notochords may be produced, but in these the elongation occurs in the first day or two after isolation, and does not continue thereafter. It seems probable that it is brought about by the continuance of gastrulation movements in the isolate, rather than by the processes which normally cause the elongation of the chorda in post-neurula stages. This suggestion is confirmed by the fact that when the isolates are made from late gastrulae or neurulae the notochords are normally not elongated, but form rounded masses. If the isolation has been made before the deposition of any noteworthy amount of chordal sheath, the cells become chaotically arranged. When notochords are taken from tail-bud stages, in which the sheath is present but still fairly thin, the whole isolate will swell as the individual cells enlarge, but it does not increase in length to any great extent. It seems clear, then, that the normal elongation is dependent on the enlarging cells being confined within a chordal sheath which increases in thickness *pari passu* with the swelling of the cells.

(*b*) *Effect of pH*

Fragments of chordal tissue from embryos of various ages have been isolated into the salines mentioned in Table 1. It has been shown by Holtfreter (1946) that alkaline salines reduce the cohesion between Amphibian embryonic cells, probably by bringing about a hydration of the cell contents. Since during the development of the chorda the cells become ever more closely applied to one another, until they are packed tightly together in the 'pile of coins' stage, it might be expected that their resistance to the disaggregating influence of alkalinity would gradually increase. This was in fact the case. Notochords from later stages were removed from the sheath before being placed in the solutions.

Solution a, *pH 11·2*

Notochord cells of all stages almost instantaneously burst and disintegrate.

Solution b, *pH 9·3*

Fragments from stages up to the late neurula begin to fall apart within a few minutes, and are usually completely disaggregated within 10–12 minutes. Early tail-bud chorda cells behave somewhat similarly, but by the late tail-bud the chorda has become more resistant and may require about 20 minutes for complete disaggregation.

The disaggregated cells in this solution show marked hyaline protuberances

and exhibit a wide variety of movements (see below). It is clear, however, that prolonged exposure to the solution is highly deleterious. The cell wall may become permeable, and the cell contents gradually escape through it, or it may break down entirely so that the cell disintegrates. Continuous culture of chorda cells in the medium is impossible.

Solution c, *pH 8·2*

Presumptive notochord cells from late gastrulae or neurulae of Axolotl remain firmly attached to one another for the first 24 hours in this solution, but thereafter they begin to round up into spheres, and gradually fall apart, usually becoming completely separated by 48 hours after isolation. In fragments from tail-bud stages of the same species there is some stretching of the cells and a lessening of their association, but they do not as a rule separate completely.

Tissue from *Triturus palmatus* is less resistant. Notochord cells from the late gastrula have completely dissociated in 4–6 hours, and there is not much difference in isolates from the neurula. Isolates from the early tail-bud may take as long as 30 hours for complete disaggregation, and tissue from late tail-bud stages can survive indefinitely without separation of the cells.

Solution d, *pH 7·6*

Separation of the cells is rather slower. In Axolotl material from late gastrulae or neurulae the cells begin to round up after about 30 hours, and have usually fallen apart in 55–65 hours, although small groups may remain coherent even after 4 or 5 days. Early tail-bud tissue of the same species shows a slight tendency for weakening of cellular attachment, but the cells do not usually become separate; late tail-bud material is apparently quite unaffected. *Triturus* gastrula and neurula tissue begins to dissociate after a few hours, even at pH 7·31, and the fragments are broken down into their component cells after about 24–36 hours. In this species early tail-bud material also becomes completely disaggregated, the cells swelling and bursting the chordal sheath in about 6 hours, becoming completely dissociated after about 36 hours. Late tail-bud fragments also exhibit swelling of the cells, but these do not become completely separate.

Solution e, *pH 7·1*

This is considered the 'normal' culture medium. The cells from all stages, of both Axolotl and *Triturus*, show no sign of disaggregation.

Solution f, *pH 6·4*

This is the mildest acidic solution used. Notochord cells differentiate perfectly in it.

Solution g, *pH 5·5*

Chordal cells can differentiate moderately well in this solution, becoming converted into a fairly normal polygonal shape. Both this solution and the last

are very effective in bringing about the re-aggregation of cells which have been dissociated in alkaline solutions.

Solution h, *pH 4·6*

Some isolated notochord cells succeed in differentiating into a polyhedral shape, but about 70 per cent. of them remain spherical and unvacuolated. Apparently the cytoplasm is solidified at this very acid pH and differentiation prevented.

Solution i, *pH 3·2*

The cells remain strongly attached to one another, and look healthy under the binocular dissecting microscope, but no differentiation occurs.

Summarizing these results, it may be seen that the chordal cells tend to become dissociated at any pH greater than 7·3. *Triturus* material is always more affected by a given treatment than Axolotl, and in both species younger tissue is more easily dissociated than older. It should be noted that all these experiments were carried out with the cells on a glass surface, to which they readily adhere; there is some evidence (Deuchar, unpublished) that disaggregation of tissues takes place much less readily when the tissue lies on agar.

(*c*) *Effect of calcium ions and detergents*

In view of the well-known importance of calcium for the maintenance of intercellular membranes in many forms of marine eggs, modifications of solution *c* (pH 8·2) were prepared in which calcium was either omitted entirely, or added in twice, four times, or eight times the normal concentration. (In the last two solutions some precipitation occurred.) Presumptive notochord cells isolated in these modified solutions disaggregated just as readily as in the unmodified solution of the same pH, although in the higher concentrations of calcium there was some suggestion that hyaline bulges were less often formed by the isolated cells.

Solution *c* was also modified by the addition of the detergent sodium lauryl sulphonate. In this way the surface tension, which in the original solution was measured (by the height of the column in a capillary) as 70·3 dynes per cm., was lowered in one case to 66·8 dynes and in another to 56·5 dynes per cm. Neither of these solutions caused any noticeable change in the behaviour of isolated fragments of notochord.

(*d*) *Mobility of isolated cells*

Cells from the chordal region of late gastrulae and neurulae, when isolated from one another by alkaline solutions, exhibit the types of mobility which have been well described by Holtfreter (1946). It is easiest to study these movements if the chorda cells are dissociated in pH 9·6 and then transferred to pH 7·6, when they remain viable and mobile for long periods.

The cell movements are associated with the formation of hyaline bulges, in

which the external surface (plasmalemma) is lifted off from the underlying plasmagel and separated from it by a fluid ectoplasmic layer.

The present observations on the movements of chordal cells fully confirmed Holtfreter's account of the behaviour of early embryonic cells in general, and there is little which it is necessary to add to his description. It is worth noting that in some cases in which a cell was ruptured so that the whole endoplasm escaped, it could be seen that the isolated cell-wall continued to exhibit bending and flowing movements for many minutes before finally disintegrating; similarly, isolated masses of cytoplasm from which the cell membrane had been lost may remain coherent and show gliding and rotary movements for a similar period.

(*e*) *Differentiation of isolated notochord cells*

If cells, isolated by alkali, are transferred to normal Holtfreter saline, the hyaline bulges disappear and the plasmalemma (cell membrane) becomes once more closely adherent to the internal cytoplasm. If several such cells lie near together, they will re-aggregate into a coherent tissue. If, however, such a cell is quite isolated, tissue formation is impossible, but nevertheless such a cell may proceed with its histological differentiation. That is to say, it becomes swollen, vacuolated, and polyhedral. As might be expected, differentiation of this kind is observed more frequently, and progresses more normally, when the cells are taken from older neurulae than when they come from the archenteron roof of a late gastrula. But it is clear that even from an early stage, single individual cells are capable of carrying out a more or less normal histogenesis. Such isolated cells do not, however, attain as large a size as they would do within the embryo.

DISCUSSION

Examination of developing notochords has shown that the most noticeable changes in the early period (from late gastrula till early tail-bud) can be summarized as an ever-increasing closeness of contact between the cells. These are at first more or less spherical and only touch one another at a few points. By the neural plate stage they have become polyhedral, and fit together so that any one cell is in direct contact with other cells over the greater part of its surface. Further closeness of contact could only be achieved by increasing the proportion of surface to volume of the individual cells; and this is what actually occurs, the cells departing markedly from equi-dimensionality. Such a departure, with the concommitant relative increase in surface, might be achieved by each cell becoming very long and narrow, assuming the shape of an elongated spindle. If the chorda cells were to develop in that way, they could only form a chorda of normal shape if they became arranged parallel to one another in a bundle, with the long axes of the cells lying in the direction of the chordal axis. In such an arrangement the cells in the middle of the bundle would be in contact all round with other cells, but those lying at the surface of the bundle would have a considerable part of their surface exposed to the ambient intercellular medium.

There would thus be considerable differences between the superficial and more deeply lying cells, and one might on general grounds suspect that such an arrangement would be unstable. Such considerations may provide some explanation for the fact that the developing chordal cells attain an increase in the proportion of surface to volume by adopting an alternative type of shape. They become exceedingly flat disks, each of which occupies the whole, or nearly the whole, cross-sectional area of the chorda. Thus each cell has only a small part of its surface (the rim of the disk) exposed to the ambient medium, while all the rest is in contact with other cells; and all the cells are very similarly placed in this respect. The arrangement would seem to be the optimum for ensuring the greatest possible cell-to-cell contact together with similarity between all cells.

After the 'pile of coins' stage, differentiation proceeds by the expansion and vacuolization of the cells, which again assume a polyhedral shape. It is perhaps possible that these changes also should be regarded as fundamentally a matter of increasing the proportion of cell-surface; but now this goes in such a way that the surface is too large for the amount of cytoplasm available, so that vacuoles are formed and the cell gradually transformed into what is little more than a large bag of cell-membrane filled with a clear sap.

During this stage another constraint has entered into the system, namely that provided by the notochord sheath, which is deposited around the developing chorda. It will be shown in another paper (Mookerjee, 1953) that the chorda is compressed by this sheath, and impeded by it from expanding in diameter, so that the greater part of the cell expansion becomes exhibited as a growth in length of the organ as a whole. It is presumably the constriction by the sheath which makes it impossible for the enlarging cells to retain their arrangement in an orderly column, and forces them once more to become irregular polyhedra.

The increasing closeness of contact between the developing chorda cells is reflected in their behaviour when exposed to alkaline solutions. Whereas the archenteron roof of the late gastrula is rapidly disaggregated, it requires a longer and longer exposure to bring about complete separation of the cells from older stages up to that of the 'pile of coins'; and thereafter the resistance to dissociation is still stronger. It is, however, not clear whether these facts are evidence that the cells are gradually becoming more and more sticky with respect to one another, or whether they depend merely on the increasing area of contact between cells whose stickiness remains unaltered.

It is noteworthy that *Triturus* tissue is more easily disaggregated than that of Axolotl, although, as Holtfreter (1945) has shown, the presumptive ectoderm of the latter shows considerable sensitivity to abnormal salines, being easily stimulated to develop into neural tissue.

The experiments reported here have done little to clarify the nature of the cohesive forces between the notochord cells. The concentration of calcium ions in the medium seems to be without effect on them. The surface-active agent sodium lauryl sulphonate was also ineffective. Holtfreter, who, like several other

authors, has laid great stress on the importance of the cell surface in morphogenesis, has discussed such phenomena in terms of surface tension (e.g. Holtfreter, 1944). There is no doubt, however, that the surface membrane of embryonic amphibian cells possesses a certain rigidity, and the concept of surface tension cannot be applied to it in any strict sense. Stableford (1949) has also presented evidence that surface tension as such is not an important force during early morphogenesis. At present, however, one cannot specify any more precisely the nature of the forces at work.

It is important to realize that, although the morphogenesis of the notochord can be very largely accounted for in terms of an increasing closeness of surface contact between the component cells, this process is not at all essential for cellular differentiation. This is demonstrated by the fact that single isolated presumptive chorda cells can continue their normal histogenesis and finish up as large vacuolated polyhedral cells, which differ from normal chorda cells only by a certain falling short in size. Such cells were, of course, determined, at least to some extent, at the time of isolation; and the present experiments give no information on the mechanism of this initial step. But the fact that differentiation can occur in isolation shows conclusively that the continuance of histological differentiation does not depend on any sort of interaction between contiguous cells. Thus it becomes rather implausible to invoke, as a possible explanation of it, a progressive immobilization of certain molecular species on the cell surface as has been suggested by Weiss (1947, 1949).

SUMMARY

1. The cellular changes during the early development of the amphibian notochord have been described. They consist essentially in an increase in the area of contact between contiguous cells. After the cells become closely packed, the area of contact is still further increased by a change to a disk-like shape, which causes an augmentation in the proportion of cell surface to volume. The disks are arranged on top of one another like a pile of coins, forming a tissue in which all the cells have a similar (and very small) proportion of their surface in contact with the noncellular ambient medium.

2. The later development of the notochord involves the conversion of the cell contents into cell membrane, the cells becoming large in volume but filled mainly by vacuoles containing a clear sap. At the time this process is beginning, a chordal sheath is being laid down around the cylindrical notochord, and it seems that this impedes an expansion in thickness and causes most of the enlargement to take the form of an increase in length.

3. As development from late gastrula to neurula proceeds, isolated fragments of presumptive chorda become more resistant to the disaggregating influence of alkaline salines. Rather unexpectedly, *Triturus* tissue was always more easily disaggregated than Axolotl. The isolated cells exhibit the forms of movement already described by Holtfreter for other tissues of the amphibian embryo.

Calcium ions and a detergent (sodium lauryl sulphonate) were without effect on the disaggregation.

4. Single isolated presumptive chorda cells from late gastrulae or neurulae can proceed with their normal histogenesis, becoming fully differentiated vacuolated and polyhedral cells, although remaining somewhat smaller than normal.

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An Experimental Study of the Development of the Notochordal Sheath

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WITH ONE PLATE

INTRODUCTION

THE prime object of the studies to be described here was the investigation of the origin and epigenetic relations of the notochordal sheath. In addition, some data were collected concerning the effects on the development of the embryo of the removal of the notochord or its substitution by some other elongated strand of material, such as human hair. Many of the phenomena encountered in the latter studies have recently been described by Kitchin (1949) and Mookerjee (1952), so that it is with questions relating to the notochord sheath that we shall be chiefly concerned.

It has been generally accepted up to the present time that the sheath is a secretory product of the notochord. This view is based on the descriptive studies of a number of comparative anatomists who dealt with the formation of the sheath and the origin of the vertebral column. Of the older workers, one may mention Hasse (1892), v. Ebner (1896), Klaatsch (1895), and Schaunisland (1906). In more recent times Gadow (1933) came to the conclusion that the sheath is formed by the notochord cells, and so did Goodrich (1930) in his *Studies on the Structure and Development of the Vertebrates*. H. K. Mookerjee (1935) raised doubts as to Gadow's interpretation of the development of the vertebral column, but did not question his views about the origin of the sheath. Tretjakoff (1927) was not satisfied by the prevailing ideas; he argued that the outer layer of the sheath (the *elastica externa*) was of mesoblastic origin, but he still held that the inner fibrous layer was secreted by the chorda.

From an entirely different point of view, the sheath has been interpreted by Studnička (1913) and Held (1921) as mesenchymal in origin. Williams (1942) also found reasons to support the view that the sheath originates from the adjacent sclerotomal tissues. In his studies on the self-differentiating powers of isolated anlagen Holtfreter (1939) found that chorda cells removed from early embryonic stages never developed a sheath. He concluded that the sheath-forming materials originate from the extra-chordal mesoblastic cells. The experiments to be described here fully corroborate this view, and further show that

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transplanted fragments of chorda can induce the laying down of a sheath by the mesoblastic cells in other regions of the body.

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MATERIALS AND METHODS

Embryos of *Triturus alpestris*, *T. palmatus*, and axolotl were used. Operations were carried out in Holtfreter solution, whose pH was kept at 7 by phosphate buffer. Healed embryos were transferred to $\frac{1}{10}$ Holtfreter. All culture solutions were made up with 0·05 per cent. sodium sulphadiazine.

The excision of the notochord was carried out through the ventral wall of the embryo. This was placed with its dorsal side downwards in a shallow depression in a wax operating dish and a long incision made from anterior to posterior on the ventral surface. This was gradually deepened through the endoderm, until the archenteron cavity was reached. The two halves of the embryo were allowed to fall away from one another, and the notochord removed through the gap thus left open. In this way the whole chorda, or any part of it, can be excised with a minimum of damage to the neural plate or to the more lateral mesoderm. After the operation the two sides of the embryo were carefully brought back into contact, and the embryo left on its side for healing, which was usually quite successful; in cases in which healing was incomplete and yolky material protruded through the wound, death usually occurred within a few days. The excised chordal material could be carefully freed of adherent mesodermal cells after its removal, and this was always done to the fragments which were to be used as isolates or transplants.

In some embryos suitable lengths of human hair, sterilized by boiling in distilled water for 15 minutes, were substituted for the excised chorda. In most cases these moved from their original position during later development, or were even completely extruded from the embryo; but in some specimens they remained in place, occupying precisely the position from which the chorda had been excised.

The embryos were fixed in Bouin's fluid, sectioned at 10μ, and stained in Delafield's haematoxylin.

FORMATION OF THE NOTOCHORDAL SHEATH

i. *Isolation experiments*

Pieces of presumptive notochord, carefully cleaned of any other adherent cells, were cultivated in isolation in Holtfreter solution for periods of up to 2 weeks. Isolates from stages as young as the late gastrula were able to carry out their histological differentiation nearly normally, the cells becoming vacuolated and polyhedral, but remaining somewhat smaller than they would in normal development (Mookerjee *et al.*, 1953). In isolates from the late gastrula or early neural plate stages the whole mass developed into such notochordal tissue, but no trace of a surrounding sheath was formed. The same was true with isolates from the stage with elevated neural folds (Plate, fig. A). In the normal embryo

the sheath is beginning to be laid down during the later stages of neural tube formation. Isolates from these or later stages therefore include some sheath. During their later development in saline solution no further deposition of sheath material occurs, although the notochord itself continues to differentiate normally. From early tailbud isolates one thus obtains well differentiated notochords surrounded by very thin sheaths.

If the isolated fragment is not wholly made up of presumptive chorda material, but includes the whole axis (neural tube, chorda, and somite mesoderm), a sheath develops round the chorda which is of a thickness comparable to that found in the normal embryo. Thus the failure of the sheath in pure chordal isolates is not due to the conditions of culture.

ii. *Implantation experiments*

When cleaned fragments of presumptive chorda, similar to those used in the isolation experiments just described, are implanted into other sites in the embryo, they develop sheaths whenever they are in a region containing mesoderm. This occurs not only in the trunk region of the body, but even in the head (Plate, fig. B), where there is no axial mesoderm but only head mesenchyme available. It seems that any mesodermal tissue is capable of depositing a sheath around an implanted chorda. On the other hand, in specimens in which the grafted chorda is, for part of its length, in close contact with the endodermal wall of the gut, it is found that the sheath is lacking in just that region.

iii. *Notochordectomy experiments*

Notochords were excised in mid-yolk plug, small yolk plug, and neural plate stages, as well as from later embryos.

The fusion of the somites and the thickening of the floor of the neural tube seem to be straightforward consequences of the absence of the chorda; they have been described not only by Kitchin (1949) and Mookerjee (1952), who removed the chorda surgically, but also by Lehmann (1934, 1937) and Cohen (1938), who suppressed chorda development by means of lithium. An attempt has been made to discover how far the effects are purely mechanical results of the absence of a central strand by substituting a relatively inert material (human hair) for the excised notochord. Only in comparatively few embryos did this remain in place during later development. In these specimens the hair effectively held apart the two rows of somites and prevented their fusion, and the neural tube then had its normal shape, with a thin floor (Plate, fig. C). The embryos, however, did not elongate appreciably better than those from which the chorda was simply removed without anything being substituted for it. The sheath of the notochord is absent round the implanted hair.

When the developing chorda is excised from younger stages (late gastrulae) the results are quite different (Mookerjee, 1952). The mesoderm mantle is at this time still plastic in its developmental capacities, and the missing organ is replaced. Fig. D of the Plate shows examples of embryos in which it is certain that

the presumptive notochord was removed from at least the greater part of the body, but in which a chorda of nearly normal size has been formed by regulation. The regulated chorda is always accompanied by its sheath.

Whenever a regulated notochord is found it is always accompanied by somitic mesoderm, though the converse is not true and somites may be found without notochord. This is of course what would be expected on Yamada's (1940) theory that the chorda represents a 'high' level on a gradient on which somites are the expression of a 'lower' level. The capacity of the mesoderm to regulate and produce a notochord after the original anlage has been excised gradually disappears during the early neural plate stages, and has vanished by the time the neural folds are well marked (Mookerjee, 1952). These results demonstrate that sheath formation never occurs without the presence of the notochord cells.

The embryos in which notochords have been reconstituted in this way can carry out a fair degree of elongation in the tail-bud and later stages, but are always considerably shorter than control embryos. This is probably not caused by any qualitative defect of the regulated notochords; in particular it is worthy of remark that the notochordal sheath, which as we shall see probably plays an important role in the mechanism of elongation, is quite normally formed. There is, however, a quantitative deficiency in the regulated notochords, which are considerably reduced in cross-sectional area. It is probable, in fact, that they are from the beginning smaller than normal in total volume, and that this is the basic reason for the comparatively slight elongation of the embryos.

DISCUSSION

The isolation experiments reported here fully confirm the view that the notochord does not form a sheath when other mesodermal tissues are absent. The transplantation experiments demonstrated that this layer is laid down around notochords which are placed among a typical mesoderm, but not around inert substances such as human hair even when the latter are in the position normally occupied by the chorda. The evidence therefore seems to be conclusive that the notochordal sheath is secreted by non-notochordal mesoblastic cells, and that this secretion is induced by contact with the chorda.

As was pointed out in the Introduction, this conclusion is contrary to the view of the origin of the sheath which has been usually accepted by comparative anatomists. It renders necessary a reconsideration of the relation between the structure of the fully developed sheath and its mode of secretion. The usual view at the present time may be presented in the words of Goodrich (1930): 'At an early stage the notochordal epithelium secretes a thin covering membrane in which intercrossing elastic fibres become differentiated. This is the *elastica externa*. Next is secreted, also by the notochordal epithelium, an inner and usually thicker fibrous sheath' (the *elastica interna*). By the notochordal epithelium Goodrich refers to the outer layer of cells in the differentiating notochord (cf. Mookerjee *et al.*, 1953). Since, as we have seen, the sheath is not formed

by the notochord itself but by cells which lie outside and around it, one cannot consider that the outermost layer of the sheath was the first to be secreted and the inner ones were laid down at successively later periods. Moreover, such an idea does not take into account the fact that the diameter of the notochord increases considerably after the sheath first appears, so that the first-formed sheath would have to be very considerably stretched if it were to accommodate the material which eventually fits inside it.

Soon after the sheath is first formed the notochord can be pushed out from within it, leaving the sheath isolated like an empty sausage skin (Plate, fig. E). It is a thin tough membrane with a glistening surface, and is similar in structure to the more external layers (elastica externa) of later stages. By the time the sheath has thickened, the inner layer (elastica interna) is formed of thicker fibres and has a dull surface. Since the sheath is laid down from the outside, one must conclude that the glistening outer layer of the early stage does not simply persist in the same condition and constitute the similar-seeming layer of later stages, but that on the contrary it gradually becomes converted into more fibrous material, while a new glistening layer is laid down outside it. It seems probable that this transformation from the glistening to the dull condition is a result of active physiological processes, which also rearrange the material so as to increase the diameter of the sheath and thus accommodate the growing notochord.

The increase in diameter of the sheath does not, however, passively keep pace with the growing volume of the notochord. This is shown by the fact that isolated fragments of presumptive chorda, which are not clothed with a sheath, increase in diameter relatively more, and in length less, than they would do normally. The elongation of the notochord, in fact, seems to be a direct result of its confinement within a relatively inelastic sheath, which forces the increase in volume to take this form. This plays an extremely important role in the development of the embryo as a whole, since the extension of the dorsal axis of the body is dependent on the growth in length of the notochord.

SUMMARY

1. Pieces of presumptive notochord of amphibian embryos, carefully cleaned of other mesodermal cells, fail to form notochordal sheath when cultivated in isolation, but a sheath is formed around them when they are transplanted to any other site in the embryo in which they come in contact with mesoblastic cells. The sheath is absent from those regions of transplanted notochords which are in contact with non-mesodermal tissues such as endoderm.

2. No sheath is formed around pieces of human hair placed in the position from which the notochord has been excised.

3. If the presumptive notochord material is excised from late gastrulae of *Triturus*, a new notochord may be formed by regulation from the more lateral mesoderm. The regulated notochords are always provided with a sheath, and conversely the sheath is absent whenever the notochord is missing.

4. It is concluded that the sheath is laid down by extra-chordal mesoblastic cells, which are induced to carry out this secretion by contact with the notochord. It is pointed out that this contradicts the conventional view held by most comparative anatomists, who have attributed the secretion of the sheath to the external layer of notochord cells (the 'notochordal epithelium').

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EXPLANATION OF PLATE

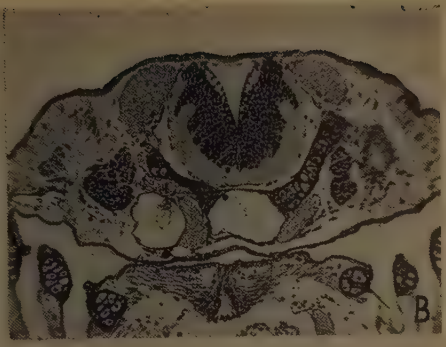
A. Differentiation of notochord cells from a neural fold stage after 12 days' cultivation in Holtfreter saline. Note the absence of a notochordal sheath.

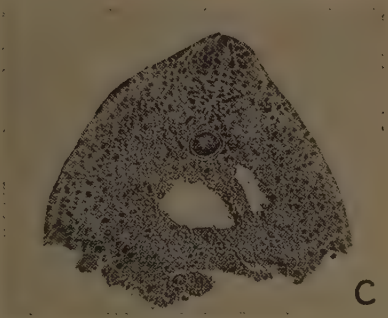
B. Embryo with notochord grafted in neural plate stage to the left of the main axis. Note the prominent sheath formed around the grafted notochord.

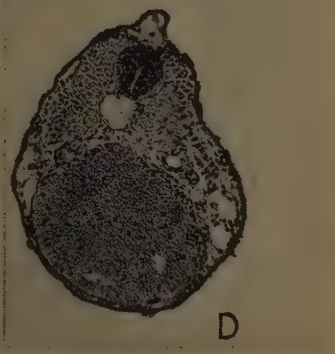
C. Cross-section of dorsal axis of an embryo in which a hair had been substituted for the notochord at an early neural plate stage. Note the normally shaped neural tube.

D. Section of an embryo to show the regulated notochord with a sheath.

E. Living notochord from a young larva. The cellular notochord has been removed from the sheath of the upper part of the notochord, and lies to the right of it.







S. MOOKERJEE

The Development *in vitro* of the Embryonic Pineal Body of the Fowl

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WITH TWO PLATES

INTRODUCTION

THE behaviour *in vitro* of the pineal body has been studied by several observers. Kasahara & Nagai (1933) cultivated the pineal body of embryonic or adult rabbits and young or adult rats as hanging drop cultures, and described the type and shape of various cells in the zone of outgrowth. The pineal body from 15 days old rats were successfully cultivated by Martinovich (1951) and prolonged cultivation of the pineal body of young rats was successfully achieved by Milković (1953).

The capacity for differentiation *in vitro* of the pineal body of the embryonic fowl has not yet been studied. Previous investigations have shown that various organ rudiments when cultivated *in vitro* undergo further normal differentiation and organization. Thus Strangeways & Fell (1926 *a* and *b*) investigated the development of the undifferentiated limb-bud and of the early embryonic eye. Fell (1928) cultivated the isolated otocyst of the embryonic fowl, and Fell & Robison (1929) the embryonic chick femur. D. H. Strangeways (1931) studied the development of the hair of guinea-pig embryos *in vitro*. Differentiation of submandibular and sublingual glands was investigated by Borghese (1950). Moscona & Moscona (1952) observed the development of the embryonic chick pituitary gland. All these organ rudiments continued their development *in vitro*.

The present investigations have shown that the isolated pineal body of 4- and of 9–12-day chick embryos possesses the capacity for self-differentiation when explanted *in vitro*.

MATERIAL AND METHOD

For these experiments 52 pineal bodies either of 4 or of 9 to 12 days old chick embryos were used. The explants were cultivated by Fell's watchglass technique on a plasma coagulum consisting of 5 drops of fowl plasma and 4 drops of extract from 9 to 11 days old chick embryos. Watchglasses placed in Petri dishes, containing moistened absorbent cotton-wool, were kept in the

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incubator at 37° C. Every 2nd or 3rd day the explants were transferred to fresh medium.

After various periods of cultivation the explants were fixed in either Zenker's or Bouin's fluid, and serial paraffin sections were stained with haematoxylin and eosin.

A series of rudiments of the normal pineal body, ranging from the 4th to the 21st day of incubation, were fixed and sectioned for comparison with the developing explants in tissue culture.

NORMAL DEVELOPMENT OF THE PINEAL BODY

The normal development of the pineal body is briefly described in order to follow more easily the development of the pineal body rudiment in tissue culture.

Patten (1927) recorded that the development of the pineal body in the chick embryo begins after 50–55 hours of incubation as a small median evagination in the roof of the diencephalon, and according to Mihalkovics (1874) it appears during the second half of the 4th day of incubation. I observed a small but distinct evagination after 72 hours of incubation.

On the 4th day the appearance is the same as on the 3rd, but the evagination is somewhat larger; at this stage the epithelial buds are not yet separated from the evagination. Sections show that the wall of the evagination consists of an undifferentiated ependymal layer continuous with the ependyma of the brain roof (Gladstone & Wakeley, 1940). The nuclei of the high columnar cells lie near the basement membrane. Two types of cells soon appear: small round cells, which border the surrounding mesenchymal tissue, and high columnar cells, facing the lumen (Mihalkovics); many of the latter are in mitosis (Plate 1, fig. 1).

During the 5th and 6th days the primary evagination gives rise to hollow epithelial buds (Plate 1, fig. 2), which gradually lose their connexion with the primary evagination. According to Mihalkovics the number of the follicles increases until the 12th day. In pineal bodies obtained from 8–12-day chick embryos (Plate 1, figs. 3 and 4) the follicles are surrounded by loose mesenchymal tissue which at the surface of the organ is condensed to form a capsule, surrounding which are a number of capillaries. This capsule connects the pineal body with the dura mater on one side, and on the other the pineal body is linked with the roof of the brain by the pineal stalk. The lumen of the pineal stalk may become obliterated and separate from the diencephalon (Gladstone & Wakeley, 1940). The data concerning detailed histological structure is rather scanty. At this stage, according to the description of Mihalkovics (1874) and Studnička (1905), the follicles are lined by two types of cells: high columnar ependymal cells 20–22 μ in height extend between the basement membrane and the lumen of the follicle with basal situated nuclei; they are surrounded by one or more layers of small cells. Intercellular borders are not sharp (Plate 1, fig. 5). The lumen of the follicles contains a thin coagulum with cell-detritus and a few free, detached cells. From the inner ends of some of the cells rounded protoplasmic processes protrude into

the lumen and may be regarded as a secretion or as degenerative material being extruded from the cytoplasm (Gladstone & Wakeley, 1940).

In late embryos (Plate 1, fig. 6) the follicles diminish in size, are much closer together, and in many of them the lumen is much reduced or has disappeared. In the adult the organ consists of solid lobules separated by septa (Bargmann, 1943).

GROWTH AND DIFFERENTIATION OF THE PINEAL RUDIMENT *IN VITRO*

Material for explantation was in the first place taken at the 4-day stage, when the evagination is slightly longer and larger than at the 3rd day, but when epithelial buds have not yet appeared. In order to avoid damaging the primordium, which is delicate and rather small, a little piece of the adjacent diencephalic tissue covered by epidermis was usually removed as well (Text-fig. 1A). In some explants the epidermis was eliminated, but there was no apparent difference in the development of the anlage explanted with or without the epidermis. The paraphysis develops as a small evagination (Dexter, 1902) from the brain roof in front of the velum transversum; it was never included in the explant.

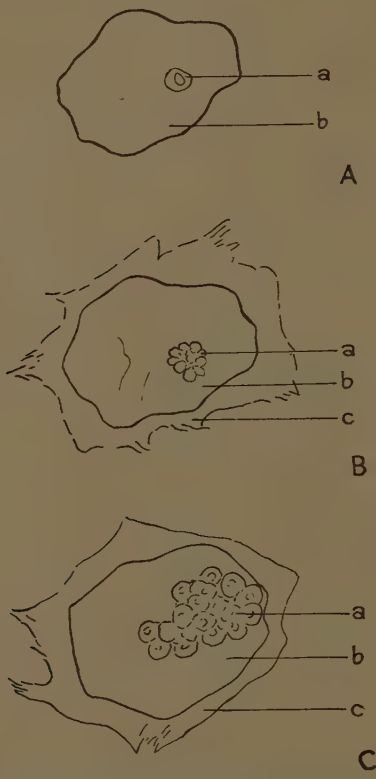
Forty-eight hours after explantation the rudiment had enlarged somewhat, and several epithelial buds had appeared around the primary evagination, so that the living anlage had the shape of a blackberry (Text-fig. 1B). Histological sections showed that epithelial buds were becoming detached from the evagination (Plate 2, fig. 7), and the explants appeared identical with normal pineal rudiments of 6-day embryos.

After the first transfer to fresh medium the tissue did not spread so much on the surface of the clot, and in consequence the explants became rounder and less transparent, so that it was difficult to observe further transformation of the shape during life.

After 4 days' cultivation, however, the pineal body was sometimes distinguishable in the surrounding mesenchymal and diencephalon tissues as of a group of follicles resembling in shape a bunch of grapes; the anlage was larger than at 48 hours (Text-fig. 1C). In many explants the histological differentiation followed a normal course; the follicles increased in number and numerous mitoses could be seen, especially in the high columnar cells next to the lumen (Plate 2, fig. 8), although some cultures did not develop so well. The size of the rudiment *in vitro* corresponded to that of a pineal body of an 8-day embryo *in vivo*, so that up to this stage no retardation in growth-rate was noticed.

After 7 to 10 days of cultivation (Plate 2, fig. 9) the histological differentiation of most of the explants was normal. Two types of cells could be distinguished: high columnar cells, extending from the basement membrane to the lumen, and small round cells near the bases of the former; many mitoses were present. The high columnar cells showed round protoplasmic protrusions into the lumen, like a secretion product, and the lumen of some follicles contained a thin clotted

mass; fibrocytes formed thin septa between the follicles. The pineal body was smaller than the normal corresponding rudiment *in vivo*, the follicles were much closer together, and their lumina smaller.



TEXT-FIG. 1. Camera lucida drawings of the pineal rudiment from a 4-day chick embryo; A, on the day of explantation; B, after 48 hours *in vitro*; C, after 4 days *in vitro*. Magnification ×40. *a*, pineal body anlage; *b*, surrounding tissue; *c*, zone of outgrowth.

In more advanced cultures, *after 13 to 17 days in vitro*, the retardation in growth was still more marked; the histological structure of the explant was almost identical with the normal pineal body of a 17–21 days old embryo (Plate 2, fig. 10), but the follicles were less numerous. In some cultures the central region was degenerate, but elsewhere the tissue remained healthy.

From the surrounding tissue which was explanted together with the anlage of the pineal body there developed brain tissue, plexus chorioideus, and epidermis, in so far as their primordia were included in the explant. In the explants from which the epidermis was not removed there developed a round epithelial

structure consisting of several layers of epidermal cells, enclosing a mass of keratin (Plate 2, fig. 11). As mentioned above, the epidermis has no influence on the differentiation of the pineal body, which developed in the same way whether the epidermal anlage was present or not. If the post-velar arch was included in the explant the plexus chorioideus usually developed very well, often forming deep folds at one end of the explant and sometimes spreading over the entire surface of the tissue (Plate 2, fig. 10); its folds were covered with a single layer of columnar cells, which were stained a metachromatic violet with haematoxylin. Part of the brain anlage included in the explant sometimes degenerated completely and disappeared, while at other times the layers of nerve-cells differentiated nicely.

Another group of explants was obtained from 9–12 days old chick embryos in which the pineal bodies were surrounded by their own capsule. The rudiments were grown until the day which corresponded to the hatching time *in vivo*, and were then fixed and sectioned. Histological sections showed that the follicles increased in number and underwent a perfect histological development; they were lined by an inner layer of columnar cells and several external layers of small round cells, and the lumina often contained a thin coagulated mass and a few desquamated cells. Thin bundles of connective tissue-fibres ran between the follicles from the capsule to the centre of the organ. The total size of the pineal body increased, but it was still much smaller than the normal body at the same stage of development (Plate 2, fig. 12).

DISCUSSION

From these experiments it is evident that the isolated rudiment of the pineal body of the chick embryo differentiated almost normally in tissue culture and from a small primary evagination many follicles lined by specialized epithelium developed. During the first 4 days of cultivation the rates of differentiation and growth were normal, so that the 4 days old rudiment after 4 days of cultivation corresponded to the 8-day stage *in vivo*. During further cultivation, however, although differentiation was almost normal, the growth-rate was much diminished, so that the size of 4-day rudiments cultivated *in vitro* for 7–10 days was much less than at the corresponding stage *in vivo*. The reason for this retardation of growth is unknown; it may have been due to the inferior nutrition of the cells resulting from the absence of a vascular system in the explants. Although in general the histological differentiation was remarkably normal in the cultures, the follicles were more closely packed together at this stage than in the corresponding normal pineal body of 11–14-day embryos and were more like those from chicks near hatching. This was probably due, not to an acceleration of tissue-differentiation, but to compression of the follicles against one another owing to the retardation of the growth-rate. In the older cultures obtained from 4 days old embryos the retardation of the growth was still greater and the explants were several times smaller than the normal organ at the same stage. The

cultures obtained from 9–12 days old embryos were also much smaller at the end of cultivation than their 21-day controls *in vivo*, but they again differentiated at an almost normal rate.

SUMMARY

1. The pineal bodies of 4 and of 9–12 days old chick embryos were cultivated by Fell's watchglass technique.

2. The rate of differentiation was almost normal, but the growth-rate was retarded *in vitro*.

3. The pineal body of 4 and of 9–12 days old embryonic fowl therefore possesses a capacity for self-differentiation which is independent of either blood-supply or of endocrine stimulation.

ACKNOWLEDGEMENTS

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I particularly want to thank Dr. H. B. Fell (Director of the Strangeways Research Laboratory, Cambridge, England) for her kind consideration in checking my paper and making helpful suggestions.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Section of the normal pineal body from an embryo at the 4th day of incubation, showing the evagination. *a*, epidermis; *b*, mesenchymal tissue. Bouin; haematoxylin and eosin. Magnification ×500.

FIG. 2. Section of the normal pineal body from a 6-day embryo. The epithelial buds are beginning to separate from the evagination. *a*, epidermis; *b*, mesenchymal tissue. Bouin; haematoxylin and eosin. Magnification ×500.

FIG. 3. Section of the normal pineal body from an 8-day embryo. The follicles are numerous. *a*, pineal stalk. Bouin; haematoxylin and eosin. Magnification ×200.

FIG. 4. Section of the normal pineal body of a 12-day embryo. Bouin; haematoxylin and eosin. Magnification ×100.

FIG. 5. The same anlage as in Fig. 4, showing follicles at higher magnification. Two types of cells are seen and there is a thin coagulum inside some of the lumina. Bouin; haematoxylin and eosin. Magnification ×200.

FIG. 6. Section through the normal pineal body of the newly hatched chick. In many follicles the lumen has disappeared. (Zenker; haematoxylin and eosin. About ×100.)

PLATE 2

FIG. 7. Section through a 4-day pineal body after 48 hours' cultivation *in vitro*. This stage corresponds to the normal control in Fig. 2. Zenker; haematoxylin and eosin. Magnification ×640.

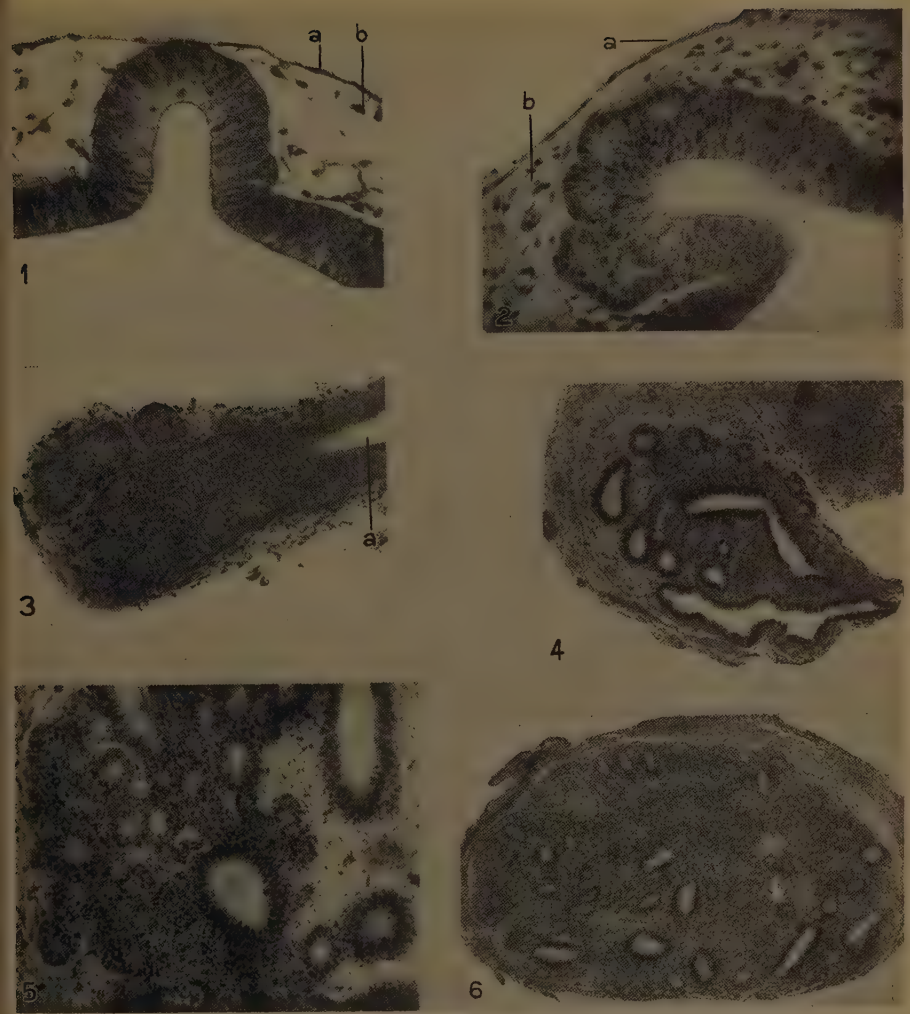
FIG. 8. Section of a 4-day culture of the pineal body from a 4-day chick embryo. Note the numerous mitoses. Compare with Fig. 3. *a*, pineal stalk. Bouin; haematoxylin and eosin. Magnification ×200.

FIG. 9. Section of a 4-day pineal body cultivated *in vitro* for 7 days. Zenker; haematoxylin and eosin. Magnification ×200.

FIG. 10. Section of a pineal body from a 4-day embryo, cultivated for 17 days *in vitro*. The culture is much smaller than the corresponding pineal body *in vivo* at hatching time. Bouin; haematoxylin and eosin. Magnification ×200.

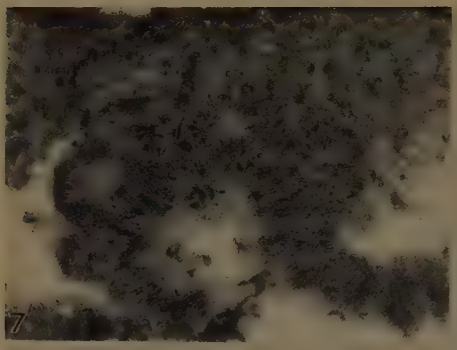
FIG. 11. Section of a pineal body explanted at the 4-day stage and cultivated for 14 days *in vitro*. Note the advanced histological development of the epidermis with keratinization in the centre of the epidermal islet *a*. Bouin; haematoxylin and eosin. Magnification ×500.

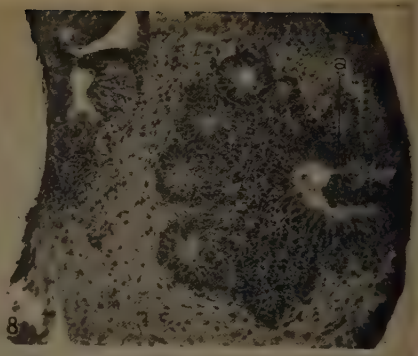
FIG. 12. Section of a pineal body explanted at the 10th day of incubation, and cultivated for 11 days. Bouin; haematoxylin and eosin. Magnification ×200.

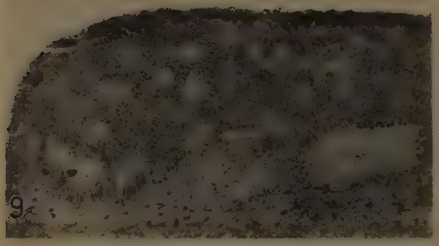


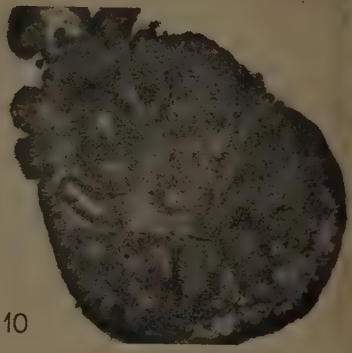
B. VIDMAR

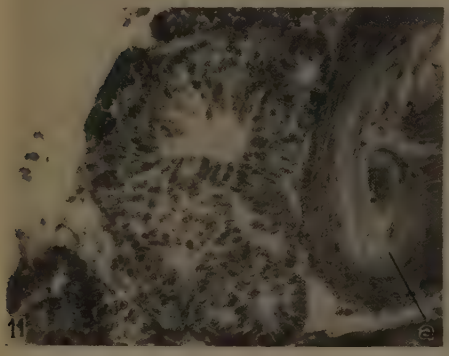
Plate 1

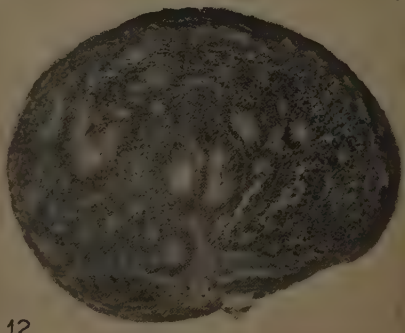












B. VIDMAR

Plate 2

Erratum

In the paper by R. M. Clayton, 'Distribution of Antigens in the Developing Newt Embryo' on page 38, line 26 from the top, *qualities* should be *quantities*.

Part 3, September 1953

INTERNATIONAL SYMPOSIUM ON THE CELLULAR BASIS OF DIFFERENTIATION, STOCKHOLM, SEPTEMBER 1952

Part 4, December 1953

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